EMOTIONAL AND SOMATIC DISTURBANCES FOLLOWING MILD TRAUMATIC BRAIN INJURY

By

Selina Eckert

July 2015

A Dissertation Presented to the Faculty of
Drexel University College of Medicine
in partial fulfillment of the Requirements for the Degree of
Master of Science

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Acknowledgements

I would like to thank all of the people who were an integral part of the completion of this thesis. First I would like to thank my mentor, Dr. Ramesh Raghupathi, for his guidance and assistance during my time at Drexel. It is under his direction that I was able to complete this work.

I would also like to thank my committee, Dr. Rodrigo España and Dr. Megan Detloff, for their support and guidance. They both helped me not only with the science but also with developing my professional life in and out of school, providing encouragement and showing interest in my professional development at the easiest and the hardest parts of my education at Drexel. It is their support, in addition to the support of the others I mention here, who helped me to complete my thesis work.

I also need to thank the various members of the Raghupathi lab for their help, teaching, friendship, and support in the lab. I want to especially thank Laura Krafjack, Lauren Hanlon, and Rupal Prasad. I learned a lot from all of you, and I hope to keep in contact wherever we all end up.

Outside of the lab, I need to thank the other people who provided their expertise for me personally as well as for this project directly. First I would like to thank all the administrative staff at Drexel's Biomedical Graduate Studies program for their help with the paperwork and technical problems I faced while I was here as well as Dr. Jed Shumsky for his help and guidance, particularly during my last year here. I would also like to thank Dr. Melanie Elliott at Jefferson University for her willingness to instruct me and help with troubleshooting the facial allodynia testing in the mice. I could not have learned this technique without her expertise and generosity. I also need to thank Dr. España for his voltammetry expertise and collaboration in running the dopamine experiments with Jessica Shaw. Jessica also put in a large number of hours in running those experiments, and we would not have had the opportunity to study the dopamine changes following single injury without her time and experience. Finally, I need to thank Dr. Detloff for her instruction in von Frey testing and exercise training as well as the use of her equipment to complete these studies.

Finally, I would be remiss if I did not thank my family and friends for all of their support and love. I could not have gotten this far without you, and I know I can count on you for whatever is to come. Special thanks go to my parents who always have my back, my sister, Becca, and brother, Josh, who are always on my side, and my friends both here and far away who supported and encouraged me through the hardest times. You may never know how important you all have been to completion of this degree, and I love you guys.

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ABSTRACT

Emotional and Somatic Disturbances following Mild Traumatic Brain Injury
Selina Eckert
Supervisor: Ramesh Raghupathi

Two of the most common symptoms following mild traumatic brain injury (mTBI) are post-traumatic headache (PTH) and depression, yet these symptoms are understudied in preclinical literature. Research has suggested a link between depressive symptoms and altered dopamine signaling and between PTH and depression. To study these connections, I first used male and female mice in a model of single mTBI and evaluated them for depressive-like behavior using the forced swim and tail suspension tests, changes in post-traumatic pain using von Frey testing, and evoked dopamine signaling using in vivo fast scan cyclic voltammetry. I found that at 4 and 8 weeks post-injury, brain-injured female mice had an increase in post-traumatic pain, and male brain-injured mice had an increase in depressive behavior in the forced swim test and displayed increased evoked extracellular dopamine following cocaine inhibition of the dopamine transporter. These results suggest changes in dopamine kinetics and signaling in brain-injured male mice which may explain the observed increase in depressive-like behavior; additionally, dopamine signaling does not appear to influence onset of PTH in mice following mTBI. Athletes represent a population of people who are at risk for sustaining repetitive mild traumatic brain injuries (rmTBI) in a short amount of time. A large

number of studies have shown that exercise strengthens the brain, yet no studies utilize exercised animals, instead only using exercise as a therapeutic intervention. In the second part of the project, I used a model of rmTBI in both exercised and unexercised mice to examine the effect of pre-injury exercise on development of depressive-like behavior and facial allodynia. I observed that rmTBI decreased depressive-like behavior and increased threshold of periorbital mechanical stimulation in brain injured male mice. In brain-injured female mice, rmTBI decreased time immobile compared to sham injured female mice, and periorbital threshold in unexercised female mice decreased over time. These results are contrary to previously published reports, and this may be due to methodological issues. However, these results demonstrate the sex-specific effects of rmTBI on development of both emotional and physical symptoms, and this study provides the groundwork for continuing studies using exercised mice as a more relevant model of athlete rmTBI.

CHAPTER ONE

REVIEW OF THE LITERATURE

1.1 EPIDEMIOLOGY OF TRAUMATIC BRAIN INJURY

1.1.1 Epidemiology of mild traumatic brain injury

Traumatic brain injury (TBI) is one of the leading causes of death and disability in the United States; in 2010 there were 2.5 million TBI-related emergency department visits, hospitalizations, and deaths (McArthur et al., 2004, Langlois et al., 2006, Centers for Disease Control and Prevention, 2014). Approximately 5.3 million Americans are living with TBI-related disabilities, and the cost of TBI annually in the United States, including medical costs and costs associated with lost productivity, is estimated at $60 billion (Langlois et al., 2006, Fann et al., 2009). Most TBIs occur in children aged 0 to 4, individuals aged 15 to 24, or those older than 65 to 75 years of age, and these occur largely due to motor vehicle accidents, assaults, and falls (Arciniegas et al., 2005, Langlois et al., 2006). Sports-related TBIs also account for a large number of TBIs occurring annually, particularly in the adolescent and young adult age groups, and it has been suggested that hospital estimates of TBI following sports injuries are low as only injuries involving loss of consciousness are included (Langlois et al., 2006).

Approximately 75-85% of all TBIs are mild (mTBI), and there are between 1.6 and 3.8 million sports-related TBIs occurring annually, highlighting the importance of studying mTBI in athletes (Langlois et al., 2006). It has been reported that 60% of retired football players receive at least one mTBI and 25% sustain multiple mTBIs over the course of their career (Bazarian et al., 2005, Langlois et al., 2006, Guskiewicz et al., 2007, Mihalik et al., 2013). However, reported incidences of mTBI may be an underrepresentation, as this does not include patients who receive care outside of the hospital, are not diagnosed with a TBI, or who do not seek treatment for their injury,

suggesting that mTBI is a much larger issue than reported in the literature (Langlois et al., 2006). However, the effects of repetitive mTBI (rmTBI) have been brought to public attention following recent media and scientific reports highlighting events such as increased suicide and depression among retired professional athletes and increases in symptoms such as depression, anxiety, and substance abuse, among others, in both adults and adolescents (Guskiewicz et al., 2007, Semple et al., 2015).

Additionally, while men are two times as likely to sustain a TBI, women tend to experience a greater number of symptoms and are a greater risk for developing persistent symptoms such as memory or attention deficit, disturbances of sleep, headache, vertigo or dizziness, irritability, depression, anxiety, and apathy (McCauley et al., 2001, Bazarian et al., 2005, Langlois et al., 2006, Centers for Disease Control and Prevention, 2014). These symptoms may be transient or chronic; 80-90% of patients experience symptom resolution spontaneously within the first 10 days post-injury, and approximately 15-50% of patients experience gradual recovery by three months-post-injury (Arciniegas et al., 2005, Spira et al., 2014). However, 40% of patients are reported to experience symptoms three to six months post-injury, and up to 20% experience symptoms beyond the first six months (Arciniegas et al., 2005).

1.1.2 Diagnosis of mTBI

Current guidelines through the National Institutes of Health list a number of factors involved in diagnosis of TBI including scores on the Glasgow Coma Scale (GCS), on which higher scores correspond to milder injury, length of any loss of consciousness, length of any amnesia, evaluations of speech and language, cognitive and neuropsychological testing, and imaging tests (Eunice Kennedy Shriver National Institute

of Child Health and Human Development, 2013). Mild TBI may be further defined by a clinician as a loss of consciousness less than 30 minutes, GCS score of 13-15 at 30 minutes post-injury, and post-traumatic amnesia lasting less than 24 hours (Blostein and Jones, 2003, Arciniegas et al., 2005). The majority of patients, approximately 80-100%, will present with headache, slowed thinking, impaired memory and attention, or a combination of these symptoms (Arciniegas et al., 2005, Barkhoudarian et al., 2011). A physical examination including evaluation of primitive reflexes, neurological examination, and neuropsychiatric assessment is also used to evaluate degree of injury (Blostein and Jones, 2003, Arciniegas et al., 2005). Cognitive assessments may include timed tests for function including attention and information processing, memory function, such as tests for encoding and retrieval, and executive functioning, and this may involve use of tools such as the Frontal Assessment Battery or Behavioral Dyscontrol Scale (Arciniegas et al., 2005). In some cases, neuroimaging techniques may be used to evaluate extent of physical injury, but these techniques may not be sensitive enough to detect structural and functional abnormalities (Arciniegas et al., 2005, Powell et al., 2008, Barkhoudarian et al., 2011).

Post-concussive syndrome (PCS) may develop as a result of mTBI and is diagnosed based on persistence of physical, cognitive, and emotional or behavioral symptoms past 2 weeks, such as memory or attention deficit, disturbances of sleep, headache, vertigo or dizziness, irritability, depression, anxiety, and apathy; post-concussional disorder may also be diagnosed following DSM or ICD-10 criteria (McCauley et al., 2001, Arciniegas et al., 2005, Bazarian et al., 2005, Leddy et al., 2012). Pre-existing physical and emotional conditions may be aggravated by a TBI; therefore, a

patient's prior medical history can be used as an important part of assessment and continuing evaluation of symptoms (Lane and Arciniegas, 2002, Arciniegas et al., 2005). PCS symptoms may continue past 2 weeks in approximately 10% of athletes and past 3 months in approximately 33% of non-athlete patients (Leddy et al., 2012).

1.1.3 Behavioral problems associated with mTBI

Mild TBI may result in persistent cognitive, emotional, and physical deficits that can have a high impact on patients' quality of life and ability to work or participate in daily activities (Langlois et al., 2006). Pathological changes resulting from the secondary injury may lead to long-lasting functional deficits in neurological function such as working memory deficit, loss of concentration, amnesia, and reduced processing speed (Ghajar, 2000, Arciniegas et al., 2005). These cognitive symptoms are often the focus of study in preclinical and clinical literature, and they may appear in either the acute or chronic post-injury period and persist for up to years after injury, depending on the severity of injury (Bales et al., 2009, Pontifex et al., 2009). Cognitive symptoms post-TBI are categorized into three groups: problems with attention and processing speed, memory, or executive function, and problems with memory are the most commonly reported symptoms (Bales et al., 2009). Attention processing includes such symptoms as difficulty concentrating, becoming easily distracted, difficulty with multitasking, and increasing time to complete a given task, and these symptoms may be related to dysfunction in cortical and subcortical pathways such as striatum, thalamus, and reticular activation as well as a potential role of dopamine pathways (Bales et al., 2009). Tests for attentional processing following TBI include the Symbol Digit Modalities Task and Digit Symbol Coding, and TBI patients perform poorly on these tasks compared to the general

population (Bales et al., 2009). Damage to the hippocampus, a structure which has been shown to be sensitive to secondary events such as cytotoxicity, may lead to the reported memory problems, though spatial memory problems have been observed without hippocampal neuronal loss, indicating that cell loss does not fully explain cognitive symptoms (Lyeth et al., 1990, Milman et al., 2005, Bales et al., 2009). Finally, dysfunction of executive control is thought to be the most disabling cognitive symptom due to its potential to increase dysfunction in other areas of cognitive function such as verbal memory, and, like the other categories of cognitive symptoms, severity and persistence of problems with executive function has been shown to increase with increased number of injuries (Bales et al., 2009, Pontifex et al., 2009).

A large number of emotional symptoms have been associated with mTBI, and these symptoms are generally most severe early after injury and resolve within a few weeks in the majority of patients (Arciniegas and Wortzel, 2014). Some common symptoms in both the early and the late post-injury periods include depression, secondary mania, anxiety, affective lability, irritability, disinhibition of behavior, restlessness and agitation, aggression, and apathy (Arciniegas and Wortzel, 2014). Pre-existing emotional and behavioral conditions may be exacerbated by mTBI, and there is a high degree of comorbidity between emotional symptoms such as depression, anxiety, and aggression which can complicate treatment and recovery from mTBI (Jorge and Starkstein, 2005, Arciniegas and Wortzel, 2014). Emotional symptoms may also increase comorbidity of physical symptoms, such as post-traumatic pain in women (Greenspan et al., 2007).

Physical symptoms include some of the symptoms commonly associated with mTBI such as headache, vision problems, vertigo and dizziness, nausea, and vomiting

(McCauley et al., 2001, Arciniegas et al., 2005, Bazarian et al., 2005, Barkhoudarian et al., 2011). These symptoms appear early after injury and typically resolve within the first few weeks, but some of these symptoms may persist up to years after injury in up to 20% of patients (Arciniegas et al., 2005).

Finally, it has been reported in a number of clinical studies that increasing the number of mTBIs to three or more significantly increases the severity and number of symptoms reported by the patient (Mannix et al., 2013, Spira et al., 2014). This may include increased mild cognitive impairment and higher levels of emotional distress, post-mTBI symptoms, and reduced attention and performance in rapid discrimination tasks, and this is reflected in fMRI studies showing functional impairments (Bailes et al., 2013, Spira et al., 2014).

1.1.4 Mechanisms of mTBI

TBI is caused by either blunt or penetrating force applied to the brain and occurs in two stages: primary and secondary injury. Primary injury refers to the mechanical injury that occurs at the time of impact, while secondary injury is the more prolonged cellular response following the primary mechanical injury.

Primary injury can occur in a number of ways, making each injury unique, but these injuries share a set of common types of forces applied to the brain. Two of these are contact and inertial forces. Contact forces occur either when an object strikes the head or when the head strikes a surface. Inertial forces, or acceleration forces, involve the movement of the head without an object striking the head or head striking a surface (Meaney and Smith, 2011). In mTBI, inertial forces play the largest role in primary injury.

Inertial or acceleration forces acting on the brain generally consist of both linear and rotational acceleration due to the head and neck motions that may occur at the time of impact (Meaney and Smith, 2011). Linear acceleration has been measured as it relates to transient increases in brain pressure, and it has been suggested that this increase in pressure may lead to neurological dysfunction relative to the amount of pressure experienced by the brain (Gurdjian et al., 1961, Meaney and Smith, 2011). Additionally, studies using human skulls filled with gelatin have determined some of the physical parameters relating to initial injury, such as the relationship between linear acceleration and head injury, leading to development of an injury tolerance curve called the Wayne State Tolerance Curve (Yoganandan and Pintar, 2004, Raymond et al., 2009, Meaney and Smith, 2011). Rotational acceleration is also an important force in mTBI. This type of acceleration leads to shear forces that occur more easily in the brain compared to other tissues because of the organization of the brain (Prange et al., 2000, Meaney and Smith, 2011). A number of studies have demonstrated that these shear forces are the largest contributing force to injury in mTBI, and this type of force also contributes largely to loss of consciousness at any injury severity (Takhounts et al., 2003, Meaney and Smith, 2011).

1.1.5 Pathophysiology of mTBI

Secondary injury following the primary mechanical injury is the leading cause of in-hospital deaths after TBI, and the pathological changes which occur may lead to long-lasting functional deficits observed in mTBI (Ghajar, 2000). The secondary injury may include neuron death or dysfunction, depending on severity, axonal damage, astrocyte and microglial reactivity, formation of a glial scar in cases of focal injury, and infiltration

of leukocytes in regions where the blood brain barrier is compromised, though these changes are generally considered to be reversible or transient following mTBI (Ghajar, 2000, Barkhoudarian et al., 2011). Additionally, decreases in glucose metabolism have been observed using positron emission tomography, and these are believed to last for a short duration following mTBI (Barkhoudarian et al., 2011). Following rmTBI, a decrease in alternative energy sources in the brain, suggestive of altered metabolism such as that seen after mTBI, was observed initially after injury as well as at 15 days post-injury, an effect not seen after single mTBI, and this decrease persisted for up to 45 days (Barkhoudarian et al., 2011).

1.1.6 Dopamine changes following TBI

A number of changes in dopamine signaling have been observed following TBI, but this has only been examined following moderate to severe TBI. Clinically, it has been observed that severe TBI reduces dopamine transporter (DAT) and D2 receptor binding in the striatum and cerebellum at approximately 5 months post-injury (Donnemiller et al., 2000). Additionally, pharmacologically increasing dopaminergic neurotransmission using methylphenidate, amantadine hydrochloride, or bromocriptine in the clinic has shown some benefit, particularly for cognitive symptoms, supporting the idea that TBI may either reduce dopamine release, increase dopamine reuptake, or alter signaling in some combination of these effects (Bales et al., 2009).

1.1.7 Consequences of mTBI

A number of acute effects of mTBI have been described such as behavioral changes, impaired memory and attention, headache, and unsteadiness (Arciniegas et al., 2005, Barkhoudarian et al., 2011). Additional mTBIs in the context of rmTBI have been shown

to increase symptom severity in all areas of affected function and increase recovery time of symptoms (Barkhoudarian et al., 2011, Spira et al., 2014).

Long-term effects of mTBI include a number of increased health risks, such as an increase in the risk of developing binge drinking, epilepsy, depression, dementia, and Alzheimer's disease as well as an increase in risk of death when compared with the general population (Langlois et al., 2006, Barkhoudarian et al., 2011). The risk of lifetime depression also increases as the number of injuries increases, as has been demonstrated in several studies in retired athletes, veterans, and military personnel (Didehbani et al., 2013, Mannix et al., 2013, Spira et al., 2014).

1.1.8 TBI treatment and management

Treatments are aimed at assisting patients in returning to normal daily living as well as preventing further injury. Education is an important part of the treatment process, providing an understanding of typical symptoms and time course for resolution as well as informing individuals about the possibility of long-term deficits, and it is thought that this education may decrease likelihood of developing post-mTBI symptoms due to potential emotional or psychological factors which have the potential to influence onset of symptoms (Arciniegas et al., 2005, Defrin et al., 2010). This education, provided for patients, family, friends, employers, and possibly insurers, can provide validation of the patient's experience, particularly following mild injury, and allow for individualized recovery plans to succeed (Arciniegas et al., 2005).

Treatment of TBI addresses the range of presenting symptoms, and in some cases, treatment of comorbid or emotional symptoms may alleviate other symptoms as well (Arciniegas et al., 2005, Kontos et al., 2012). Pharmacologic or behavioral interventions

are chosen to address specific symptoms, though some of these therapies may be overlapping. For emotional symptoms such as depression, psychotherapy or pharmacologic intervention may be used (Arciniegas et al., 2005). Nonpharmacologic interventions may also be useful for treatment of cognitive and physical symptoms. While a number of behavioral treatments may be useful, no pharmacologic interventions have been approved by the FDA, making any prescriptions off-label and requiring ongoing evaluation by clinicians. For example, treatments of cognitive symptoms after injury may involve the use of methylphenidate acutely after injury to address attention problems; methylphenidate may also be beneficial for information processing ability and mood or behavioral symptoms (Arciniegas et al., 2005, Bales et al., 2009). Other pharmacotherapies may include dextroamphetamine, amantadine, bromocriptine, or L-dopa or carbidopa, among others, again focused on alleviating cognitive symptoms (Arciniegas et al., 2005). It has been suggested that L-dopa or carbidopa may also be beneficial for symptoms of apathy and that amantadine may improve agitation, aggression, depression or affective lability (Arciniegas et al., 2005, Tan et al., 2015). However, treatments are highly individualized, as therapies that are beneficial in one case may be ineffective in another (Arciniegas et al., 2005).

1.2 ANIMAL MODELS OF MILD TBI

1.2.1 Animal models of mTBI

Several different models for mTBI have been utilized including weight drop, blast-induced injury, lateral fluid percussion, and controlled cortical impact (CCI). While the mechanism for producing the injury is different between these models, they have demonstrated similar cellular and behavioral changes in rodents. Depending on injury

severity, neurons and axons may either be injured or die, and diffuse axonal injury is observed even in mTBI; glia also experience physical damage and may react with severity-dependent degrees of activation or reactivity (Chen et al., 2003, Myer et al., 2006, Creed et al., 2011). The glial cell response tends to be more prolonged than the neuronal response and involves both a morphological change and a change in gene expression and secretion profiles that may affect surrounding neurons (Chen et al., 2003). This may contribute to conditions such as glial scarring and inflammation as well as reactive phenotypes, but secretion of factors such as nerve growth factor by astrocytes may also help support neuron regeneration and axon regrowth, illustrating the dual role of glia in response to trauma. Secondary processes observed in these models may also include cytotoxicity such as altered calcium and magnesium regulation, free radical formation, neurotransmitter excitotoxicity, and vascular disruptions (Arciniegas et al., 2005, Barkhoudarian et al., 2011).

Behavioral changes have also been observed following mTBI in rodents, and the most commonly studied behaviors are cognitive function such as learning and memory, though mood disturbances are also sometimes studied. For example, transient deficits in working memory and increased depressive-like behavior have been observed following mTBI in mice (Milman et al., 2005, Schwarzbold et al., 2010, Creed et al., 2011). Increases in anxiety-like behavior have also been observed following mTBI in both rats and mice (Schwarzbold et al., 2010, Almeida-Suhett et al., 2014).

1.2.2 Animal models of repeated mTBI

Several different model systems have been used to characterize the effects of rmTBI, and the most common are the weight drop and CCI in rats or mice. However, there is a great

degree of variation between the details of the repetitive injury models, such as location of injury, type of impactor tip, duration between injuries, and number of injuries. Additionally, the majority of studies occur only in male animals. These studies have shown that multiple injuries, typically three or more, within 24 to 72 hours apart lead to increased glial activation, axonal injury, impairment in learning, increased neuroinflammation, increased phosphoTau immunoreactivity, increased exploratory behavior, and increased depressive-like behavior (Longhi et al., 2005, Mannix et al., 2013, Ojo et al., 2013, Luo et al., 2014, Mouzon et al., 2014, Petraglia et al., 2014). However, the length of time between injuries remains a point of debate; it is unclear how much time between injuries is necessary to prevent the aggravation of symptoms. Several different researchers have reported anywhere from 24 hours to five days between injuries leading to increased cognitive or behavioral deficits or cellular damage and dysfunction while others report that any longer than 24 hours between injuries is more similar in symptoms to a single mTBI (Longhi et al., 2005, Bolton and Saatman, 2014).

1.2.3 Dopamine changes following TBI in animals

Dopamine signaling changes have been observed in animal models similar to those suggested in humans following TBI, though these have only been studied following moderate to severe TBI. Tissue levels of dopamine are altered in a region-specific and post-injury time-specific manner; increased dopamine is observed at 6 hours post-injury in striatum, between 1 and 24 hours in the hypothalamus, and at 14 days post-injury in the prelimbic and infralimbic cortex but are decreased in cortex from 1 hour up to 2 weeks post-injury (McIntosh et al., 1994, Kobori et al., 2006, Bales et al., 2009). Tyrosine hydroxylase activity is increased at 28 days post-injury in frontal cortex, and

both activity and protein levels are increased at 2 weeks post-injury in prelimbic and infralimbic cortex, possibly suggesting an upregulation of expression to compensate for a reduction in available tissue dopamine (Yan et al., 2001, Kobori et al., 2006). Finally, in a lateral injury model, DAT protein level is decreased in ipsilateral cortex at 7 days and bilaterally at 28 days post-injury, again suggesting altered dopamine signaling following TBI that may contribute to cognitive and emotional symptoms (Yan et al., 2002, Bales et al., 2009). In more recent studies examining post-TBI changes in DAT expression, it has been observed that decreased DAT occurs mostly in males, thought to be due to estrogen's role in dopamine signaling, and environmental enrichment has been shown to improve decreases in DAT to a greater degree in female animals than in male animals (Bales et al., 2009). However, despite indications that altered dopamine signaling affects post-TBI symptoms in a gender-specific manner, the beneficial or detrimental impact of each of these changes remains a matter of debate (Bales et al., 2009).

1.3 POST-TBI DEPRESSION

1.3.1 Epidemiology of post-TBI depression

Clinical depression is reported in 6 to 33% of all mTBIs within the first year post-injury, and some studies report an incidence as high as 77%, making it one of the most prevalent psychiatric conditions following TBI; depression symptoms may develop in the acute or chronic post-injury period and persist for a longer period of time than cognitive symptoms (Rapoport et al., 2003, Jorge et al., 2004, Arciniegas et al., 2005, Jorge and Starkstein, 2005, Fann et al., 2009). In fact, depression has been reported to persist in 17% of patients at three to five years post-injury (Dikmen et al., 2004, Arciniegas et al., 2005). Due to the overlap of depression and mTBI symptoms, depression is often

underreported, partially accounting for the wide variation in prevalence between studies (Kontos et al., 2012). Depression resulting from mTBI is usually most severe in the early post-injury period and tends to resolve within the first few weeks, but increasing the number of injuries or severity of injury may prolong the length of symptoms or increase severity (Arciniegas and Wortzel, 2014, Spira et al., 2014). Depression following mTBI is often experienced in conjunction with other behavioral disturbances including anxiety, substance abuse, emotional lability and dysregulated emotional expression, and aggression, and presence of these comorbid conditions may complicate treatment and recovery from mTBI (Jorge and Starkstein, 2005, Arciniegas and Wortzel, 2014). In fact, it has been reported that 76.7% of patients with post-traumatic depression experience comorbid anxiety and 56.7% exhibit comorbid aggression (Jorge et al., 2004, Jorge and Starkstein, 2005). Additionally, TBI patients who develop depression tend to have poorer cognitive performance and three times the rate of autonomic and psychologic symptoms (Jorge et al., 2004, Jorge and Starkstein, 2005, Kontos et al., 2012). Finally, depression may be associated with increased comorbid pain conditions, such as headache, and women tend to experience comorbid pain conditions, such as depression and anxiety, more often than men (Craft, 2003, Greenspan et al., 2007).

1.3.2 Assessment of post-TBI depression

Assessment of depression following TBI relies on evaluation of symptoms, similar to depression in the non-injured population (Arciniegas et al., 2005, Jorge and Starkstein, 2005). However, diagnosis may be complicated by the overlap of depression symptoms and TBI symptoms (Jorge and Starkstein, 2005, Kontos et al., 2012). DSM-based criteria appear to have the highest specificity and sensitivity to detection of post-traumatic

depression compared to other diagnostic criteria at a number of times post-injury, including acutely, at 3 months, at 6 months, and at 1 year post-injury (Jorge and Starkstein, 2005). These criteria include symptoms such as depressed mood and anhedonia, two symptoms that are more relevant to distinguishing depression from physical symptoms of TBI such as changes in sleep, appetite, or libido (Jorge and Starkstein, 2005, Fann et al., 2009).

1.3.3 Treatment of post-TBI depression

Treatments for post-TBI depression are similar to treatments for depression in the non-injured population, including pharmacotherapies, psychotherapy, or a combination. It is thought that selective serotonin reuptake inhibitors (SSRIs) are more effective and better tolerated in this population, but some researchers and clinicians recommend choosing SSRIs with short half-lives such as sertraline, citalopram, or escitalopram (Arciniegas et al., 2005). Tricyclic medications may also be used, but they tend to be less effective and carry higher risk of adverse reactions such as seizures during the acute post-injury period, and for these reasons they are not the pharmacotherapy of choice and have higher drop-out rates than other medications (Arciniegas et al., 2005, Fann et al., 2009). The efficacy of pharmacotherapy following TBI has not been well studied, but few adverse effects are reported other than in tricyclic antidepressants (Fann et al., 2009). As with treatment of other post-traumatic symptoms, evaluation may continue over treatment course on an individualized basis. Psychotherapy is also commonly used, but the efficacy of psychotherapeutic interventions restricted to treatment of depression has not been well studied due to presence and treatment of other psychological problems present after TBI (Fann et al., 2009).

1.3.4 Pathophysiology of post-TBI depression

Development of depression following TBI may result from a number of changes that occur in brain structure and function. For example, a number of neurotransmitter systems including glutamatergic, cholinergic, dopaminergic, and serotonergic systems, as well as neuroendocrine systems, have been observed to be altered, and these changes may be linked to mood and behavioral disturbances including stress responses, mood regulation, motivation, anhedonia, memory and executive function, agitation, and disinhibition (Murdoch et al., 1998, Agha et al., 2004, Jorge and Starkstein, 2005). Additionally, many of these changes have been observed in brain regions linked to different symptoms of depression including the hippocampus, mesocorticolimbic pathway, and prefrontal cortex (Jorge et al., 2004, Jorge and Starkstein, 2005). However, it remains unclear exactly how these changes influence depression as opposed to other symptoms of mTBI, once again illuminating the overlap in symptoms between different aspects of the effects of TBI.

1.4 POST-TRAUMATIC HEADACHE

1.4.1 Post-traumatic headache epidemiology

One of the most common complaints following TBI is headache (Lane and Arciniegas, 2002). Post-traumatic headache (PTH) is defined as a secondary headache in patients without prior headache disorders or the worsening of a prior headache disorder which develops within the first 7 days post-injury, though the majority of PTH cases develop within the first 1 or 2 days post-injury (Lane and Arciniegas, 2002, Linder, 2007). If the headache persists for longer than 3 months, it is considered to be chronic PTH while PTH which resolves within the first 3 months is acute PTH (Lane and Arciniegas, 2002). PTH has been reported in 30-90% of patients, and prevalence may vary with severity of injury

or time after injury; however, it remains unclear if prevalence is related to injury severity, as some studies suggest more association with milder injuries while others have found no correlation between injury severity and development of PTH (Lane and Arciniegas, 2002, Walker et al., 2005, Defrin et al., 2010, Elliott et al., 2012). The majority of patients, around 70-85%, experience symptom resolution, but the remaining 15-30% develop chronic and debilitating PTH that may greatly reduce quality of life and function (Lane and Arciniegas, 2002). There is some debate over the etiology of symptoms, such as the contribution of emotional factors including post-traumatic stress to symptom onset, but regardless of other contributing factors, four mechanisms of PTH development have been proposed. The currently proposed mechanisms are activation of peripheral pain pathways following cervical trauma at the time of injury, activation of central pain pathways due to the primary injury to the brain, activation of peripheral pain pathways leading to central pain pathway activation via anatomical or neurochemical means, and trauma-induced neurochemical dysregulation (Lane and Arciniegas, 2002). PTH may be classified as one of several different types including tension-type, which accounts for approximately 75% of cases, migraine without aura, accounting for 21% of cases, or otherwise unclassifiable, accounting for the remaining 4% (Lane and Arciniegas, 2002, Linder, 2007). Headache symptoms as a result of trauma are indistinguishable from non-traumatic headache symptoms, and for this reason many diagnostic criteria are similar between chronic headache disorders and PTH (Lane and Arciniegas, 2002).

Headache conditions, particularly studied in migraine, have been observed to increase risk of onset of depression and anxiety, two common comorbid psychiatric PTH conditions (Torelli et al., 2006). Some researchers have suggested that onset of PTH may

be related to stress or emotional conditions such as post-traumatic stress disorder, but this remains unclear (Defrin et al., 2010). Additionally, clinical and preclinical studies of pain have demonstrated an effect of sex on development of nocicpetive problems and effects of analgesia, and it has also been observed that women more commonly experience pain disorders such as headache than men (Craft, 2003). Women also experience comorbid pain conditions, such as depression and anxiety, at higher rates than men (Greenspan et al., 2007).

1.4.2 PTH diagnosis

Diagnostic criteria for PTH differ slightly between mTBI and moderate to severe TBI as well as between acute and chronic PTH (Lane and Arciniegas, 2002, Linder, 2007). Diagnostic criteria were developed in 2004 by the International Headache Society, and several characteristics are consistent regardless of type of TBI. To be diagnosed as PTH, headache must develop within 7 days of a head trauma or after regaining consciousness following head trauma (Lane and Arciniegas, 2002, Baandrup and Jensen, 2005, Linder, 2007). For acute PTH, the headache must either resolve within 3 months post-injury or the headache persists but less than 3 months have passed since the injury; similarly, for chronic PTH, headache must persist for at least 3 months after TBI (Baandrup and Jensen, 2005, Linder, 2007). Whether acute or chronic, additional criteria are specific to injury severity. Following a mTBI and in addition to the criteria specified above, the patient must fulfill at least one of three criteria including no loss of consciousness or loss of consciousness less than 30 minutes, a score on the Glasgow Coma Scale of 13 or higher, or signs and symptoms of mTBI (Baandrup and Jensen, 2005, Linder, 2007). After moderate to severe TBI, in addition to the criteria above, at least one of four criteria

must be fulfilled including loss of consciousness for longer than 30 minutes, Glasgow Coma Scale score of less than 13, post-traumatic amnesia for longer than 48 hours, or traumatic brain lesion confirmed using imaging techniques, usually CT or MRI (Baandrup and Jensen, 2005, Linder, 2007).

1.4.3 PTH treatment

Choice of treatment for PTH relies on type of headache, but many of the treatments used for general headache disorders are also used for PTH treatment (Baandrup and Jensen, 2005). PTH displaying migraine-like features is associated with central pain pathway activation, and treatments are chosen to target these features. This treatment mainly involves preventive and abortive pharmacologic interventions including calcium channel-blockers, beta-blockers, antidepressants, and anticonvulsants (Lane and Arciniegas, 2002, Arciniegas et al., 2005). In cases displaying tension-type headache, treatment of peripheral problems such as neck pain may be sufficient to reduce PTH; this may include physical therapy, biofeedback, and trigger-point injections, sometimes in conjunction with nonsteroidal anti-inflammatory drugs, antidepressants, and muscle relaxants (Lane and Arciniegas, 2002). Finally, PTH with mixed symptoms, or PTH with features of both central and peripheral pain pathway activation, may involve combined therapies addressing both types of headache (Lane and Arciniegas, 2002). In addition to pharmacologic or behavioral interventions, patients are also provided education, support, and counseling for PTH management, and if these treatments are ineffective, comorbid medical and psychological conditions are re-evaluated and treated (Lane and Arciniegas, 2002). Comorbid psychiatric conditions, including depression and anxiety, as well as medical conditions, such as sleep disorders, are known to affect experience and reporting

of pain; therefore treatment of these conditions is likely to improve PTH outcome (Lane and Arciniegas, 2002, Torelli et al., 2006, Robbins, 2013, Arciniegas and Wortzel, 2014).

CHAPTER TWO

DEVELOPMENT OF POST-TRAUMATIC PERIORBITAL ALLODYNIA AND DEPRESSIVE-LIKE BEHAVIOR AFTER A SINGLE MILD TRAUMATIC BRAIN INJURY

2.1 ABSTRACT

Two of the most common symptoms following mild traumatic brain injury (mTBI) are post-traumatic headache (PTH) and depression, yet these symptoms are understudied in preclinical literature. Research has suggested a link between depressive symptoms and altered dopamine signaling, and PTH may also be linked to depressive symptoms. Additionally, sex has been suggested to be important in development and maintenance of depression and pain following mTBI. To study these connections, I used male and female mice in a model of mTBI and evaluated them for depressive-like behavior using the forced swim and tail suspension tests, changes in periorbital allodynia using the von Frey filament test, and evoked dopamine signaling using in vivo fast scan cyclic voltammetry. I found that at 4 and 8 weeks post-injury, brain-injured female mice had an increase in post-traumatic periorbital allodynia. Additionally, male brain-injured mice had an increase in depressive behavior in the forced swim test and displayed increased evoked extracellular dopamine following cocaine inhibition of the dopamine transporter. These results suggest changes in dopamine kinetics and signaling in brain-injured male mice which may explain the observed increase in depressive-like behavior; additionally, dopamine signaling does not appear to influence onset of PTH in mice following mTBI.

2.2 INTRODUCTION

2.2.1 Animal models of depression

Evaluation of depression in animals poses a great challenge due to uniquely human symptoms of depression, such as depressed mood, low self-esteem, and suicidality, which cannot be measured in non-human animals (Kapur and Mann, 1992, Cryan et al., 2005, Deussing, 2006). Instead, animal models of depression focus on different commonly reported symptoms of depression in humans such as anhedonia and despair which may be measured using a combination of tests that have been developed for rats and mice. The most commonly used tests for depressive behavior in rodents are the forced swim test (FST) and the tail suspension test (TST), both despair-based tests, the sucrose preference test, an anhedonia-based test, and in some studies, the open field test, an anxiety-based test.

The FST was first described in 1977 and has been one of the most well-characterized and most used tests for depressive-like behavior in a number of disease models (Porsolt et al., 1977, Porsolt et al., 1978, Cryan et al., 2005). In the FST, animals are placed in a beaker of water in which they cannot touch the bottom or escape for a set amount of time while the time spent passively floating is measured. This test relies on escape behavior in rodents and evaluates amount of immobility, or time during which the animal is not actively swimming or trying to escape, as a measure of passive stress-coping strategies, also referred to as depressive-like behavior or behavioral despair (Porsolt et al., 1978, Deussing, 2006). This test has been shown to be reliably reproduced between facilities and is able to screen for antidepressant drugs; in the FST, substances effective as antidepressants decrease immobility, converting passive coping to active

coping (Cryan et al., 2005, Deussing, 2006). Additionally, by distinguishing different swim behaviors such as vertical climbing or horizontal movement (climbing versus swimming), this test can also distinguish activity of catecholinergic from serotonergic drugs (Cryan et al., 2005, Deussing, 2006).

Similar to the FST, the TST is another despair-based evaluation for depressive-like behavior in rodents (Steru et al., 1985). In the TST, animals are suspended by the tail for a set amount of time, and the amount of time the animal spends immobile is measured. Like the FST, TST is a reliable predictor of antidepressant efficacy, and in C57BL6 mice baseline immobilities are comparable between the two tests (Cryan et al., 2005, Deussing, 2006, Frye, 2011). However, some variability in immobility time exists between the two tests with treatment of certain antidepressants like rolipram and levoprotiline, imipramine treatment, or GABA receptor antagonism, suggesting that the two tests operate via two distinct mechanisms (Bai et al., 2001, Cryan et al., 2005, Deussing, 2006). For example, administration of imipramine in mice has been shown to decrease immobility in the FST at doses between 5 and 15 mg/kg while a dose-dependent decrease in immobility was observed in the TST up to 45 mg/kg (Bai et al., 2001). Another example of this difference in immobility was observed following $GABA_B$ receptor knockout or antagonism; compared to wild type or untreated mice, knockout or antagonism resulted in decreased immobility observed using the FST, but no difference in immobility was observed using the TST (Mombereau et al., 2004). It is unclear whether these differences between tests are specific to mice, as TST is typically conducted in mice but not rats, but it has been demonstrated that these differences are strain-specific between as many as 11 strains of mice (Bai et al., 2001, Cryan et al.,

2005). Additionally, these tests have rarely been conducted in female mice, so sex differences also remain unclear (Cryan et al., 2005). Despite potential differences, the TST offers several advantages over the FST including removing concern over motor issues present in some genetic and injury models, reducing any hypothermic response-induced variability that may be present in the FST, and increased sensitivity of the TST to detect SSRI activity, particularly early in treatment (Cryan et al., 2005). Despite these advantages, the TST has several drawbacks such as strain-specific problems with tail-climbing behaviors; tail-climbing animals are generally removed from analysis as they have learned escape is a possibility, therefore increasing sample size to compensate for these animals (Cryan et al., 2005).

Two reward-based tests for depressive-like behavior include the sucrose preference test and intracranial self-stimulation. These tests evaluate the human symptom of anhedonia, and both involve not only affect but also a motivational component. Animals exhibiting depressive-like behavior will show a decreased consumption of sucrose as well as decreased intracranial self-stimulation (Deussing, 2006). While both of these tests are responsive to chronic administration of antidepressants, they are not as well validated as the despair-based tests (Deussing, 2006). Finally, the open field test may be used as an anxiety-based test. In this test, animals are placed in an open, brightly lit box, and time spent along the edges versus in the center as well as crossings between zones is evaluated as a measure of anxiety (Choleris et al., 2001, Burghardt et al., 2004). This test is less well-characterized as a depression test, focusing on a single aspect of depression that may be considered an entirely separate disorder from depression or a comorbidity rather than a symptom of depression.

2.2.2 Animal models of PTH

While there are a number of preclinical models for headache disorders, only one model has been used in the context of PTH. Possible models include mechanical stimulation, pin prick, air puff, cold sensitivity, and behavioral observations. Mechanical stimulation, pin prick, air puff, and cold sensitivity measure evoked pain responses while observations of facial grooming behaviors evaluate spontaneous pain. Of these tests, mechanical stimulation and temperature sensitivity have been utilized in clinical studies; these studies have shown that individuals experiencing chronic PTH develop facial allodynia to mechanical stimulation and increased sensitivity to temperature in at least one of several painful regions of the head including the frontal region, temporal region, occipital region, and vertex or crown of the head (Defrin et al., 2010). This increased sensitivity is not observed in individuals who do not develop PTH or in uninjured control subjects.

Mechanical stimulation using von Frey filaments is the only technique that has been used in preclinical studies of PTH (Elliott et al., 2012, Macolino et al., 2014). In this method, the periorbital region of the face is stimulated using a predetermined range of von Frey filaments, and responses to stimulation are recorded. The size of the filaments used vary with species and, if other regions of the body are tested such as the forepaw, with the region of stimulation. Animals experiencing chronic pain conditions, such as PTH, exhibit development of allodynia, measured as a decrease in threshold sensitivity to mechanical stimulation, when compared to sham injured or naïve animals (Elliott et al., 2012, Macolino et al., 2014).

While only von Frey testing has been performed in preclinical studies following TBI, the headache field offers several other possibilities for testing of post-traumatic

pain. These tests have been used in models of headache including infraorbital nerve constriction and dural stimulation, two rodent models for headache and migraine. First is the pin prick test. In this test, a 24 gauge needle is bent at a 30° angle and applied twice to the face of the animal (Vos et al., 1994). This stimulation is expected to consistently produce a behavioral response in normal animals and evaluates mechanical hyperalgesia rather than mechanical allodynia. Another option is an air puff test. For air puff, a gentle air puff lasting between 1 and 4 seconds is applied to the center of the vibrissal pad 10 times bilaterally with at least 10 seconds between stimulations (Jeon et al., 2012, Dieb and Hafidi, 2013). The final test for evoked pain is cold hypersensitivity. In this test, acetone is applied to sensitive regions, as determined by prior testing, and frequency of scratching behavior is evaluated and considered to be indicative of cold allodynia (Jeon et al., 2012).

The final test for headache evaluates spontaneous pain rather than evoked pain. This test involves observation of facial grooming behaviors. Animals are recorded for 7 minutes, and the number of facial grooming episodes, such as "face wash strokes" persisting for at least 4 seconds per episode, as well as identification of fifteen different facial grooming behaviors are evaluated (Vos et al., 1994). Increases in facial grooming are thought to represent increased spontaneous pain, but due to the number of behaviors evaluated in this test, it is more complicated than those for evoked pain.

2.2.3 Dopamine involvement in depression and pain

The neurobiology of depression has focused on neurotransmitter systems such as serotonergic and noradrenergic systems, but the role of dopamine (DA) pathways in development and maintenance of depression is gaining attention (Kapur and Mann, 1992,

Nestler and Carlezon, 2006). DA is suggested to be involved in a number of depression symptoms such as anhedonia, defined as the inability or reduced ability to experience pleasure, and depressed locomotor activity, but more recent studies have suggested that altered DA function has more influence on the loss of motivation than anhedonia directly due to dysfunction of reward pathways (Dunlop and Nemeroff, 2007, Finan and Smith, 2013). In light of these depression symptoms, a few different mechanisms have been proposed for the role of DA and dopaminergic pathways in depression including reduced DA release, impaired signal transduction, altered receptor number or function, altered intracellular signal processing, or a combination of these processes (Dunlop and Nemeroff, 2007). Several animal models of depression such as learned helplessness, mild chronic stress, and behavioral despair have also demonstrated a potential role for DA dysfunction. Animals displaying learned helplessness behaviors have been observed to have depleted DA in the caudate nucleus and nucleus accumbens (NAc); additionally, pretreatment with DA agonists prevents development of this behavior while DA antagonists can prevent the action of antidepressants and increase learned helplessness behaviors (Kapur and Mann, 1992, Dunlop and Nemeroff, 2007). However, it is important to note that studies utilizing DA agonists and DA transporter or reuptake inhibitors have indicated that there is a sex-dependent increase in arousal or locomotor activity which may contribute to the decreased learned helplessness behavior; treatment with cocaine, DA D1 agonist SKF 82958, and DA uptake inhibitor GBR 12909 have been shown to increase locomotor activity in both males and female with a greater increase in females (Trampus et al., 1993, Schindler and Carmona, 2002, Monti and Monti, 2007). DA signaling is also known to function in chronic stress, one factor that

may lead to onset of depression, and in a model of mild chronic stress it has been found that D2 and D3 receptor binding is decreased in the NAc and may be reversed by antidepressant treatment (Dunlop and Nemeroff, 2007). Additionally, stress activates DA neurons in the ventral tegmental area (VTA), and this activation may become altered with chronic stress exposure, potentially becoming pathologic such as in mood disorders (Nestler and Carlezon, 2006, Finan and Smith, 2013). Using the FST for depressive behavior in rodents, it has been observed that immobility, the measure for despair, is reversed by D2 or D3 receptor agonists, the DA and norepinephrine reuptake inhibitor nomifensine, and tricyclic antidepressants while D2 or D3 antagonists increase immobility (Kapur and Mann, 1992, Dunlop and Nemeroff, 2007). Additionally, it has been shown that the antiparkinsonian drug amantadine, which may act as an indirect DA agonist by increasing presynaptic DA release, inhibiting DA reuptake, and possibly increasing density of postsynaptic DA receptors, attenuates depressive-like behavior following severe TBI in rats, though the mechanism behind the dopaminergic effects remains unclear (Sawyer et al., 2008, Giacino et al., 2012, Tan et al., 2015).

The role of DA in chronic pain conditions has not been extensively studied, but there is some evidence that DA neurotransmission may be involved in the pathogenesis of pain. It has been observed that endogenous DA within the mesolimbic system may lead to analgesia; lesions to the VTA, leading to decreased DA, have been observed to produce an increased sensitivity to pain while VTA stimulation produces an analgesic effect in rats (Sotres-Bayon et al., 2001, Finan and Smith, 2013). Additionally, it has been observed in patients with facial pain or fibromyalgia that the concentration of DA metabolites in cerebrospinal fluid is reduced (Finan and Smith, 2013). Genetic studies in

humans have observed that some haplotypes of genes involved in DA function, such as the catechol-O-methyltransferase (COMT) gene and some polymorphisms of the D2 receptor, are correlated with pain conditions and some common pain comorbidities such as depression and anxiety (Peroutka et al., 1998, Finan and Smith, 2013). The COMT gene is involved in regulation of tonic DA and is thought to be involved in pain modulation, and it has been proposed that poor tonic DA regulation may largely contribute to the persistence of chronic pain (Finan and Smith, 2013). Additionally, altered DA function has also been implicated in headache and migraine conditions. While there is still a great deal of debate and conflicting literature, it has repeatedly been shown that migraineurs exhibit DA receptor hypersensitivity and hypoactive DA signaling, that DA antagonists are beneficial for acute treatment of migraine, and that DA agonists are effective in prophylactic migraine treatment (Chen, 2006, D'Andrea et al., 2006, Akerman and Goadsby, 2007). Migraine and cluster headache patients have increased levels of platelet DA compared to healthy control individuals, suggesting that this may represent a specific phenotype in headache sufferers (D'Andrea et al., 2006). Finally, it has been observed that drugs which affect DA also affect vasodilation, one of the proposed mechanisms for headache, and that DA receptors are expressed in the trigeminal ganglion and spinal trigeminal nucleus, two structures also implicated in headache disorders, and DA receptors themselves are thought to regulate nocicpetion, vasoregulation, and autonomic responses (Chen, 2006, Akerman and Goadsby, 2007).

2.2.4 Significance

Despite rising evidence indicating the high incidence and impact of emotional and physical symptoms on patients following mTBI, the development of depression and PTH

following mTBI remain unclear. In particular, it remains unclear how depressive symptoms and PTH develop in a sex-specific manner following TBI as well as what structural and functional changes may influence development of these symptoms. Due to the lack of studies addressing these questions, this study focuses on development of depression and PTH following mTBI in male and female mice, examining the potential role of DA signaling changes in these post-traumatic symptoms.

2.3 MATERIALS AND METHODS

2.3.1 Animals

Male and female C57BL/6J mice (aged 6-8 weeks; Jackson Laboratories, Bar Harbor, ME) were used for this study. Animals received standard chow *ad libitum* and were maintained four mice to a cage in a controlled temperature environment with a 12 hour light-dark cycle. All procedures used followed guidelines set by the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and these procedures were approved by the Drexel University Institutional Animal Care and Use Committee.

2.3.2 Concussive brain injury

Mice received a single mild closed skull injury as previously published (Creed et al., 2011). Mice were anesthetized using isoflurane (1.5-2.5%; Penn Veterinary Supply, Lancaster, PA) inhalation via a nose cone. Body temperature was maintained during surgery using a heating pad set to 37°C. The scalp was swabbed with povidone-iodine and isopropyl alcohol, and mice received a subcutaneous injection of lidocaine prior to making a 1.0 cm midline rostral to caudal incision in the scalp. The periosteum was reflected and mice were placed in a standard mouse restrainer (Braintree Scientific, Braintree, MA) with the head supported by a soft foam pad so that it was level with the body. The mouse in the restrainer was then positioned beneath the cortical impact device (Custom Design & Fabrication, Richmond, VA), and a 5-mm-diameter hemispheric metal impactor tip was zeroed against the exposed skull by touching it to the sagittal suture midway between the bregma and lambda sutures. Thirty seconds after removal of

isoflurane, the impactor tip was electronically driven perpendicularly onto the exposed sagittal suture at a velocity of 5.0 m/s and a depth of 1.5 mm beyond the zero point. Immediately following injury, righting reflex was obtained by evaluating the amount of time until the mouse regained normal posture over three consecutive attempts when placed in a supine position. Following righting reflex, animals were re-anesthetized and scalp incisions sutured with 4-0 silk sutures. Sham-injured mice received the same surgical procedures without receiving an impact from the metal impactor tip. All animals were allowed to recover on a heating pad set at 37° and were returned to home cages once they became sternally recumbent. All mice received injections of Buprenex (0.05 mg/kg; McKesson, San Francisco, CA) following injury, at 2 hours, and at 24 hours post-surgery; animals for von Frey experiments received two injections: one following injury and one at 6 hours post-surgery.

2.3.3 Assessment for depressive-like behavior

In order to test for depressive-like behavior, the forced swim test (FST; Figure 2.1 A) and tail suspension test (TST; Figure 2.1 B) were used. Baselines were acquired approximately five days before injury, and animals were evaluated at 4 and 8 weeks post-injury or post-surgery. In the FST, mice are placed in a beaker of water that is deep enough that the animal cannot rest its tail on the bottom of the beaker but not filled so high that the mouse can escape. Water temperature was kept between 21 and 25°C to reduce variability of behavior (Petit-Demouliere et al., 2005). Animals were recorded for 6 minutes, and time immobile, when the mouse is not actively swimming, was measured. Paddling without movement is considered immobility, as it has been identified as the least amount of effort necessary for the mouse to remain afloat (Petit-Demouliere et al.,

2005). Time immobile is considered to be a readout for depressive-like behavior, or "despair," and immobility is measured for the last 4 out of 6 minutes of the test. Only a single trial is necessary in mice to provide a stable measurement of immobility, unlike in a rat model; therefore only a single trial was conducted at each time point (Cryan et al., 2005). In the TST, mice are suspended by the tail and recorded for 6 minutes, and time immobile is measured for the full 6 minutes (Cryan et al., 2005). Like in the forced swim test, greater immobility time indicates increased depressive-like behavior.

2.3.4 Assessment for mechanical sensitivity

In order to test for changes in mechanical stimulation and development of facial allodynia following injury, I used the ascending method of von Frey (VF) stimulation, as previously published (Elliott et al., 2012, Macolino et al., 2014). Animals were evaluated at 4 and 8 weeks post-injury or post-surgery. Mice were acclimated to the testing room for one hour before being placed in a standard plastic restrainer (Braintree Scientific, Braintree, MA) placed at a 30° angle for a maximum of 15 minutes (Figure 2.2). Von Frey filaments of sizes 0.008, 0.02, 0.04, 0.07, and 0.16 g were applied to the periorbital area 5 times bilaterally with 10 seconds between stimulations for each filament beginning with the smallest filament and progressing to larger filaments until the threshold was obtained (Elliott et al., 2012). Positive responses were recorded as head shaking, stroking the face with the forepaw, or withdrawal from stimulus, and thresholds were considered to be 3 or more positive responses out of 5 on each side, or at least 60% positive responses for the filament. All facial allodynia testing occurred before noon on the day of testing to reduce variability. Allodynic thresholds were determined as values at or below

0.04 g, or two filament sizes below 0.1 g, which is the previously published naïve baseline for male mice (Macolino et al., 2014).

2.3.5 Fast-scan cyclic voltammetry

To evaluate changes in DA signaling, *in vivo* fast-scan cyclic voltammetry was performed at the conclusion of all other behavioral tests. Mice were anesthetized with isoflurane and placed in a stereotaxic apparatus. Following surgical preparation, a carbon fiber recording electrode was lowered into the nucleus accumbens core (NAc; +1.3mm anterior; -1.3mm lateral; -4.5mm ventral) and a Ag/AgCl reference electrode was lowered into the contralateral cortex. A stimulating electrode was placed in the ventral tegmental area (VTA; -3.0mm anterior; +1.1mm lateral; -4.5mm ventral) through which DA release was evoked by application of an electrical pulse train (300 μA, 4 ms) every 5 minutes. Electrode placement is presented in Figure 2.3. The electrode potential was scanned linearly at the carbon fiber electrode from -0.4 V to 1.2 V and back to -0.4 V at a scan rate of 400 V/s every 100 ms. Electrode positions were adjusted to achieve maximal DA response. A minimum of three stable collections (within 10%) were acquired before mice were treated with 10 mg/kg i.p. cocaine (6 mg/ml) and subsequent changes in DA responses (peak height and *tau*) were monitored for a minimum of 1.5 hours following administration of cocaine. Baseline and post-cocaine estimated extracellular DA and *tau* were calculated using Demon Voltammetry and Analysis software written in Labview language (National Instruments, Austin, TX).

2.3.6 Statistical analyses

Quantifications were performed with experimenters who were blinded to injury status of the mice. Data are expressed as mean ± standard error of the mean (SEM). Comparisons

of immobility times for FST and TST as well as threshold values for VF testing were determined using repeated measures analysis of variance (ANOVA) followed by Newman-Keuls *post-hoc* if ANOVA rejected the null hypothesis. Comparisons of peak height and *tau* for voltammetry experiments were determined using factorial two-way ANOVA followed by Fisher LSD *post-hoc* if ANOVA rejected the null hypothesis. Significance for percent allodynia was determined using Chi Square analysis. Distributions of periorbital thresholds were evaluated using the Kruskal-Wallis one way ANOVA followed by a Mann-Whitney U test if ANOVA rejected the null hypothesis. Only p values < 0.05 were considered to be statistically significant.

2.4 RESULTS

2.4.1 Acute neurological responses following closed head injury

No acute or delayed mortality resulted from mild closed head injury. However, a minor fracture perpendicular to the sagittal suture appeared in approximately one third of the mice. Sham injured mice righted themselves spontaneously within 30-120 seconds following removal of anesthesia. Contrasting this, brain-injured mice righted themselves within 160-700 seconds. Brain-injured mice experienced a brief period of apnea lasting from 1-20 seconds which was not observed in sham injured mice.

2.4.2 Depressive-like behavior

Development of depressive-like behavior was observed in male but not female brain-injured mice at 4 and 8 weeks post-injury (Figure 2.4). In male mice, a repeated measures ANOVA indicated an increase in time immobile in brain-injured compared to sham injured mice in the FST [$F(1,22) = 10.85$, $p = 0.003$] but not the TST [$F(1,22) = 0.57$, $p > 0.05$]. No significant difference was observed in either test between brain-injured and sham-injured female mice (FST: [$F(1, 20) = 0.09$, $p > 0.05$]; TST: [$F(1, 20) = 0.49$, $p > 0.05$]).

2.4.3 Development of periorbital allodynia

Sensitivity to mechanical stimulation of the periorbital region was tested at 5 and 8 weeks post-injury. A significant decrease in periorbital threshold, indicative of development of allodynia, was observed at both 5 and 8 weeks post-injury in adult female brain-injured mice compared to sham injured female mice [$F(1, 10) = 14.54$, $p = 0.003$] (Figure 2.5 A). No difference was observed between male brain-injured and male sham injured mice

[F (1, 10) = 0.014, p > 0.05]. Percent of mice developing allodynia in each group is given in Figure 2.5 B. At 5 weeks, 40% of male sham injured mice displayed allodynia while 43% of brain-injured male mice displayed allodynia. At 8 weeks, 20% of sham injured male mice displayed allodynia and 43% of brain-injured male mice displayed allodynia. There was no significant difference between number of brain-injured and number of sham injured male mice which displaying allodynia at 5 weeks (X^2 = 0.0098, n = 12, p > 0.05) or at 8 weeks (X^2 = 0.69, n = 12, p > 0.05). At 5 weeks, 20% of sham injured females displayed allodynia and 100% of brain-injured female mice displayed allodynia, a difference that was significant (X^2 = 8.4, n = 12, p = 0.0038). At 8 weeks, 20% of female sham injured mice displayed allodynia while 71% of female brain-injured mice displayed allodynia, which was not statistically significant (X^2 = 3.09, n = 12, p > 0.05). Individual distributions of periorbital thresholds for each group are given in Figure 2.6. A Kruskal-Wallis one-way ANOVA indicated no significant differences between brain-injured and sham injured male mice at either 5 or 8 weeks (H(3) = 1.14, p > 0.05). In contrast, a significant group difference was observed between brain-injured and sham injured female mice (H(3) = 9.07, p = 0.028); a Mann-Whitney U test indicated that sham injured female mice had a significantly higher threshold than brain-injured female mice at 5 weeks (U = 2.5, p = 0.015) but not at 8 weeks (U = 6.5, p > 0.05).

2.4.4 Changes in dopamine signaling

Levels of evoked extracellular DA were recorded in the NAc of a subset of brain-injured and sham-injured male and female mice. A two-way ANOVA revealed no significant differences between brain-injured and sham-injured mice in either males or females at baseline using peak decay analysis for either extracellular DA concentration (p > 0.05 for

both males and females; Figure 2.9) or *tau* ($p > 0.05$ for both males and females; Figure 2.10). T-test analysis of baseline measurements showed no significant differences between brain-injured and sham injured male mice for extracellular DA concentration, $t(12) = -0.22$, $p > 0.05$, or *tau*, $t(12) = -0.02$, $p > 0.05$, and no difference between brain-injured and sham injured female mice for extracellular DA concentration, $t(12) = 1.86$, $p > 0.05$, or for *tau*, $t(12) = 0.97$, $p > 0.05$. However, following dopamine transporter (DAT) inhibition using cocaine, a significant increase in evoked extracellular DA was observed in male brain-injured mice compared to male sham injured mice [$F(1,22) = 6.53$, $p = 0.02$]. No significant difference was observed between female brain-injured mice and female sham injured mice following cocaine, but Newman-Keuls *post hoc* analysis indicated that male brain-injured mice had a significantly higher level of extracellular DA following cocaine than female brain-injured mice ($p = 0.035$). T-test analysis of post-cocaine percent change in extracellular DA indicated no significant differences between brain-injured and sham injured male mice for extracellular DA concentration, $t(11) = 1.76$, $p > 0.05$, or for *tau*, $t(11) = -0.14$, $p > 0.05$, and no differences between brain-injured and sham injured female mice for extracellular DA concentration, $t(11) = -1.97$, $p > 0.05$, or *tau*, $t(11) = -0.45$, $p > 0.05$.

2.5 DISCUSSION

Mild traumatic brain injury (mTBI) is a growing public health concern in populations including student and professional athletes as well as military personnel, and some of the most common and most understudied post-traumatic symptoms include depression and post-traumatic headache (PTH). Here, I evaluated development of depressive-like behavior and post-traumatic allodynia in male and female mice. I observed that at 4 and 8 weeks post-injury, male mice developed depressive-like symptoms in the FST but not the TST while no change was observed in female mice. Additionally, I observed that female mice developed periorbital allodynia at 5 and 8 weeks post-injury while there was no change between male brain-injured and sham injured mice. Finally, I evaluated evoked dopamine (DA) release in a subset of male and female mice and found no difference between brain-injured or sham injured male or female mice at baseline. However, following inhibition of the dopamine transporter (DAT) using cocaine, male brain-injured mice displayed a significantly greater release of DA in the nucleus accumbens compared to sham injured male mice and female brain-injured mice while no difference was observed between female sham injured and brain-injured mice.

Clinical depression is a common symptom following mTBI, occurring in approximately 6 to 33% of all mTBI patients within the first year post-injury (Rapoport et al., 2003, Jorge et al., 2004). However, few clinical studies examine men and women as separate groups, instead combining men and women in analyses of incidence and symptoms and leaving the influence of gender on emotional symptoms unclear despite evidence that sex can influence post-traumatic symptoms and development of depressive disorders (McCauley et al., 2001, Bazarian et al., 2005, Greenspan et al., 2007). These

results showed that only male brain-injured mice developed a depressed phenotype. Additionally, this result was seen only in the FST and not the TST. While typically the FST and TST are cited to produce comparable results at baseline, some studies using antidepressants have shown different results between the two tests. It has been suggested that this inconsistency is because the two tests induce immobility, or time when the animal is not engaging in escape behavior, via different mechanisms, though the responsible mechanisms remain unknown (Cryan et al., 2005, Deussing, 2006). It is possible that this model of mTBI in mice affects the function of the system responsible for immobility in the FST differently in male and female mice, leading to an increase in this behavior in male brain-injured mice but not female brain-injured mice. However, the results I observe here are consistent with previously published studies in male mice. Most other studies of depressive-like behavior in rodents following mTBI have used the FST and have reported increases in depressive-like behavior as early as 7 days post-injury and persisting out to at least 3 weeks post-injury in male mice (Milman et al., 2005, Washington et al., 2012, Higashi et al., 2014). However, the mechanisms responsible for inducing depression in these models are still under investigation.

Several animal and human studies have suggested that dysfunctional DA neurotransmission following TBI may be related to development of symptoms. In humans, reduced DAT and D2 receptor binding in striatum and cerebellum has been observed following TBI, suggesting altered function of the DA system (Donnemiller et al., 2000). Additionally, altered tissue levels of DA and altered tyrosine hydroxylase activity has been reported in animal models after TBI (McIntosh et al., 1994, Yan et al., 2001, Yan et al., 2002, Kobori et al., 2006, Bales et al., 2009). Dysfunctional DA

signaling has been implicated in development and maintenance of both depression and pain conditions, suggesting that dysfunctional DA signaling may play a role in development of these symptoms following TBI (Kapur and Mann, 1992, Chen, 2006, D'Andrea et al., 2006, Akerman and Goadsby, 2007, Dunlop and Nemeroff, 2007). I found using in vivo fast scan cyclic voltammetry that at baseline there was no significant difference in extracellular DA concentrations between brain-injured and sham injured male mice or between brain-injured and sham injured female mice. However, after administration of cocaine, a compound that inhibits DAT function, there was a significantly greater concentration of extracellular DA in male brain-injured mice. One possible explanation for these findings is that brain-injured male mice have a reduced amount of available DA after mTBI, leading to compensatory mechanisms such as a reduction in DAT number or function in order to maintain tonic extracellular DA levels. Due to these mechanisms, inhibition of DAT in these animals would lead to an accumulation of extracellular DA, as seen in these results. While further studies are necessary to validate this mechanism, such as an evaluation of DAT number or function in brain-injured male mice, a similar mechanism has been proposed and supported through fMRI studies as one explanation for the involvement of dysfunctional DA neurotransmission in major depression in humans (Dunlop and Nemeroff, 2007). In this proposed mechanism it is suggested that depressed patients have a reduction in DA release that leads to compensation such as an increase in postsynaptic DA receptors and a decrease in DAT density, ultimately resulting in increased DA signal transduction (Dunlop and Nemeroff, 2007). With this in mind, it is reasonable to suggest that the altered DA neurotransmission observed in male brain-injured mice may contribute to the

depressive-like behavior observed in the FST in this same group of animals. However, this mechanism does not account for the *tau* results I observed in which there were no significant changes in values for *tau* between groups. I would expect that if DAT number or function was decreased, *tau* would be altered to indicate deficits in DA reuptake. However, perhaps DA neurotransmission is sensitized following single mTBI in male mice, leading to the observed increase in DA release following cocaine without altering reuptake, and these changes could contribute to DA-related symptoms. For example, this type of mechanism may be observed in Parkinson's disease patients who are compulsively taking dopaminergic drugs. It has been observed that these patients show no differences in basal DA levels but display enhanced drug-induced dopaminergic neurotransmission in the ventral striatum, though uptake rates were not evaluated (Evans et al., 2006). DA sensitization has also been observed in several rodent studies of drug dependence in which increased drug-induced DA release in regions such as the NAc and ventral striatum has been observed (Robinson et al., 1988, Parsons and Justice, 1993). Substance abuse and depression, both symptoms that may occur following TBI, have been observed to be highly comorbid in clinical populations in the absence of injury, and similar mechanisms may be involved in their development (Swendsen and Merikangas, 2000, Taylor et al., 2003). If a similar mechanism is occurring in these animals as has been observed in substance abuse studies, it could explain the changes in post-cocaine DA release without altering *tau* and represent a potential mechanism for the depressive-like behavior observed here.

In addition to depression, another common post-traumatic symptom is headache, more commonly reported in women than in men (McCauley et al., 2001). PTH develops

in 30 to 90% of patients and persists long after injury in 15 to 30% of patients who develop PTH, causing significant functional impairment (Lane and Arciniegas, 2002). I used periorbital mechanical sensitivity as a model for PTH in these mice. This model is one of several models used to evaluate headache in mice and offers several advantages over other rodent headache models. First, it uses a technique that has previously been used in both clinical and preclinical TBI studies. Increased mechanical sensitivity has been observed in painful regions of the head in humans who develop PTH compared to patients who do not develop PTH or TBI-free patients (Defrin et al., 2010). Additionally, increased periorbital sensitivity has been observed in mouse models of moderate to severe TBI (Elliott et al., 2012, Macolino et al., 2014). Finally, using von Frey filaments, it is possible to observe both increased and decreased mechanical sensitivity in brain-injured mice. Contrary to this test, tests which evaluate cold hypersensitivity or use a pin prick or air puff may only be capable of indicating either an increase or a decrease in sensitivity, limiting observable changes in these mice. This may be important if a decrease in sensitivity occurs following mTBI, as this has not been previously evaluated.

In this model of PTH, I observed sex-specific development of periorbital allodynia following mTBI. All female brain-injured mice displayed allodynic thresholds at 5 weeks whereas only 43% of male brain-injured mice displayed allodynic thresholds at this time, and while the percentage of animals displaying allodynia decreased by 8 weeks in female brain-injured mice, there was no change in male brain-injured mice. This is consistent with clinical research suggesting that women are more likely to develop symptoms of headache as well as preclinical research suggesting that female animals are more likely to develop pain conditions than male animals (Craft, 2003, Greenspan et al.,

2007), supporting this model as a relevant model of mTBI in mice. When examining number of animals displaying allodynic thresholds, I observed a significant difference between sham injured and brain-injured female mice at 5 weeks but not at 8 weeks. However, using average periorbital threshold values, I found that female brain-injured mice displayed significantly reduced periorbital thresholds at 5 and 8 weeks post-injury, suggesting a development of mechanical allodynia and chronic central pain in these mice. I did not observe any change in brain-injured male mice compared to sham injured male mice. While I observed the development of allodynia in female mice as expected, the lack of allodynia in male brain-injured mice does not correspond with previous research, as it has previously been reported that male brain-injured mice develop significant periorbital allodynia up to 4 weeks post-injury (Elliott et al., 2012, Macolino et al., 2014). It is possible that this is due to injury severity, as the Elliott group used a more severe injury than in the current study, or that male mice develop periorbital allodynia earlier than 5 weeks, our earliest evaluation time, following mTBI. An assessment of earlier time points post-injury is necessary to evaluate these options, but it is unlikely that this test can be utilized earlier than 7 days post-injury due to confounding factors of post-surgical pain. Finally, while DA has been suggested to play a role in the development and maintenance of chronic pain conditions (Chen, 2006, Finan and Smith, 2013), this study does not support the involvement of dysfunctional DA neurotransmission in the development of PTH following mTBI as female brain-injured mice developed allodynia but did not experience altered DA neurotransmission

In summary, I showed here that at 4 and 8 weeks following single mTBI there is an increase in depressive-like behavior in male brain-injured mice, a finding that

coincides with altered DA neurotransmission observed using in vivo voltammetry. Additionally, I observed an increase in periorbital mechanical sensitivity in female brain-injured mice at 5 and 8 weeks post-injury. Taken together, these data support the sex-specific development of post-traumatic symptoms of depression and PTH, and while alterations in the DA pathway do not appear to affect development of allodynia, these alterations may be involved in depressive-like behavior in this model of mTBI in mice.

2.6 FIGURES

Figure 2.1: Experimental setup for depression testing. Examples of tests for depressive-like behavior including the forced swim test (A) and tail suspension test (B).

Figure 2.1

Figure 2.2: Experimental setup for von Frey testing. Mice were placed in a standard plastic mouse restrainer set at a 30° angle for periorbital mechanical sensitivity testing using von Frey monofilaments.

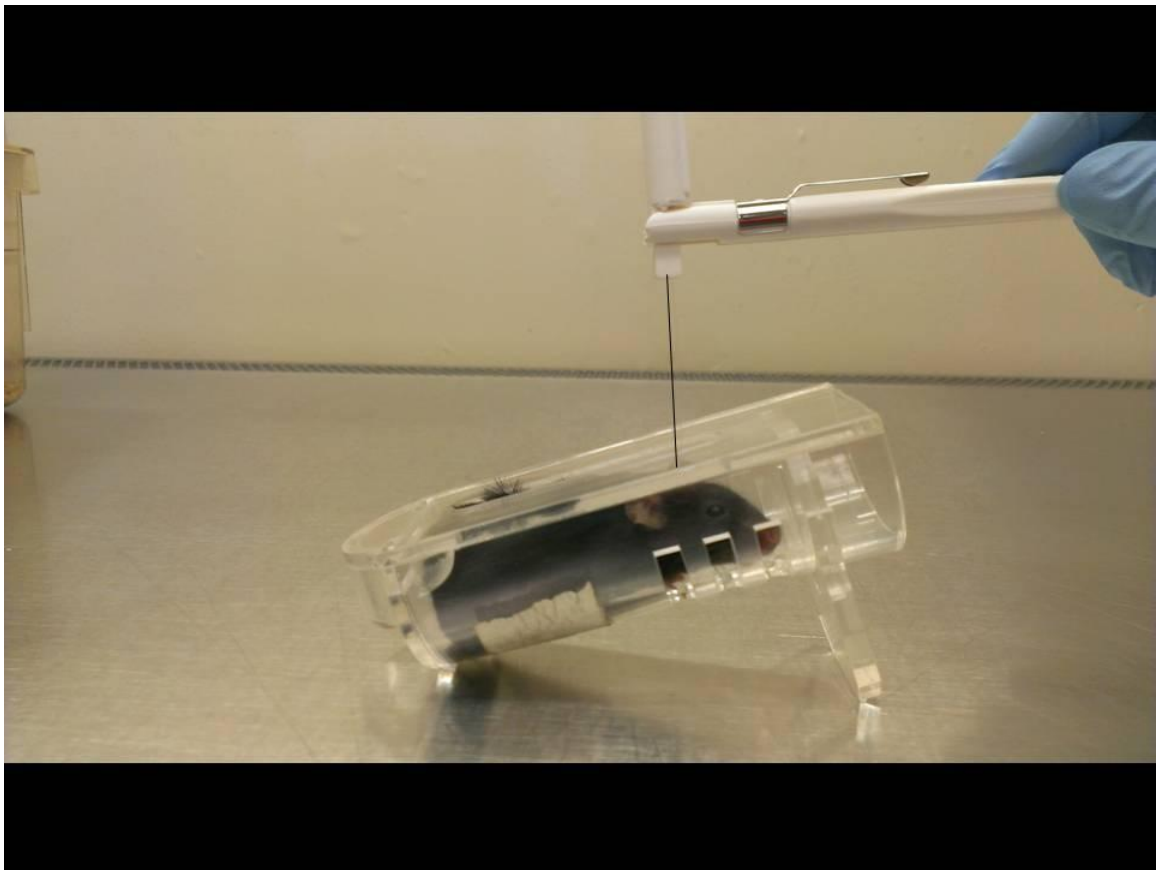


Figure 2.2

Figure 2.3: Placement of electrodes for voltammetry experiments. Stimulating electrodes were placed in the ventral tegmental area (VTA) and recording electrodes were placed in the nucleus accumbens (NAc). The template for this figure was provided by Dr. Rodrigo España.

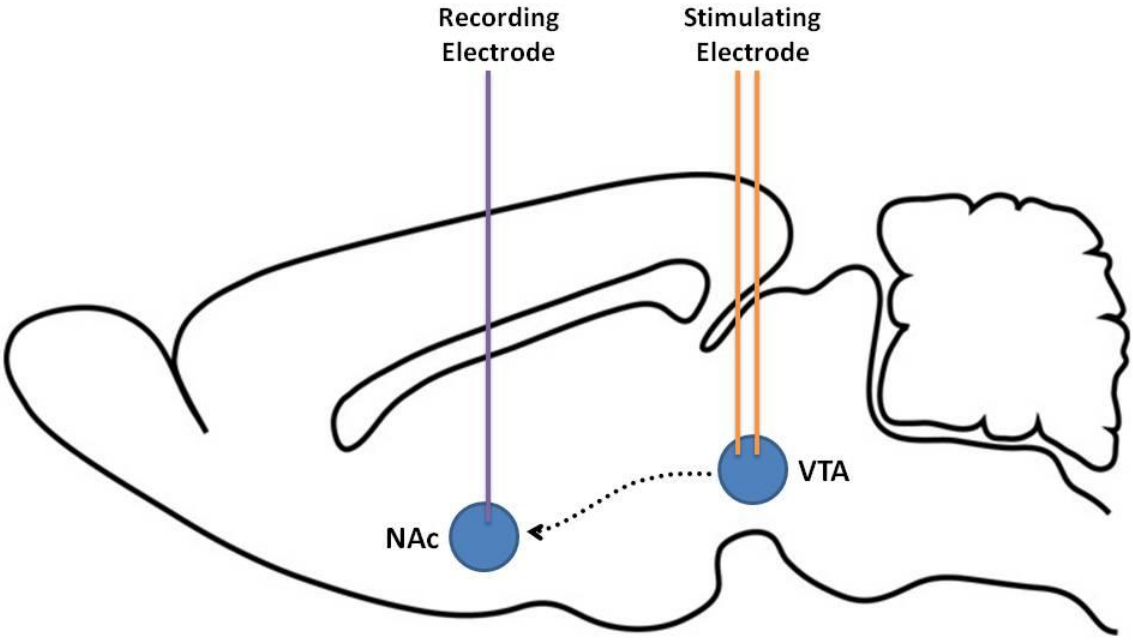


Figure 2.3

Figure 2.4: mTBI increases depressive-like behavior in male but not female mice. Mice were tested for depressive-like behavior using the forced swim test (A) and tail suspension test (B) at 4 and 8 weeks following a single mTBI. Repeated measures ANOVA indicated that brain-injured male mice had significantly greater time immobile than sham injured male mice ($p = 0.003$) while there was no significant difference between sham injured and brain-injured female mice ($p > 0.05$) for the forced swim test. There were no significant differences between sham injured and brain-injured male mice ($p > 0.05$) or between sham injured and brain-injured female mice ($p > 0.05$) in the tail suspension test. Figures are pictured as group mean ± Standard Error of the Mean (SEM); *$p < 0.05$ compared to sham injured mice; NS = not significant.

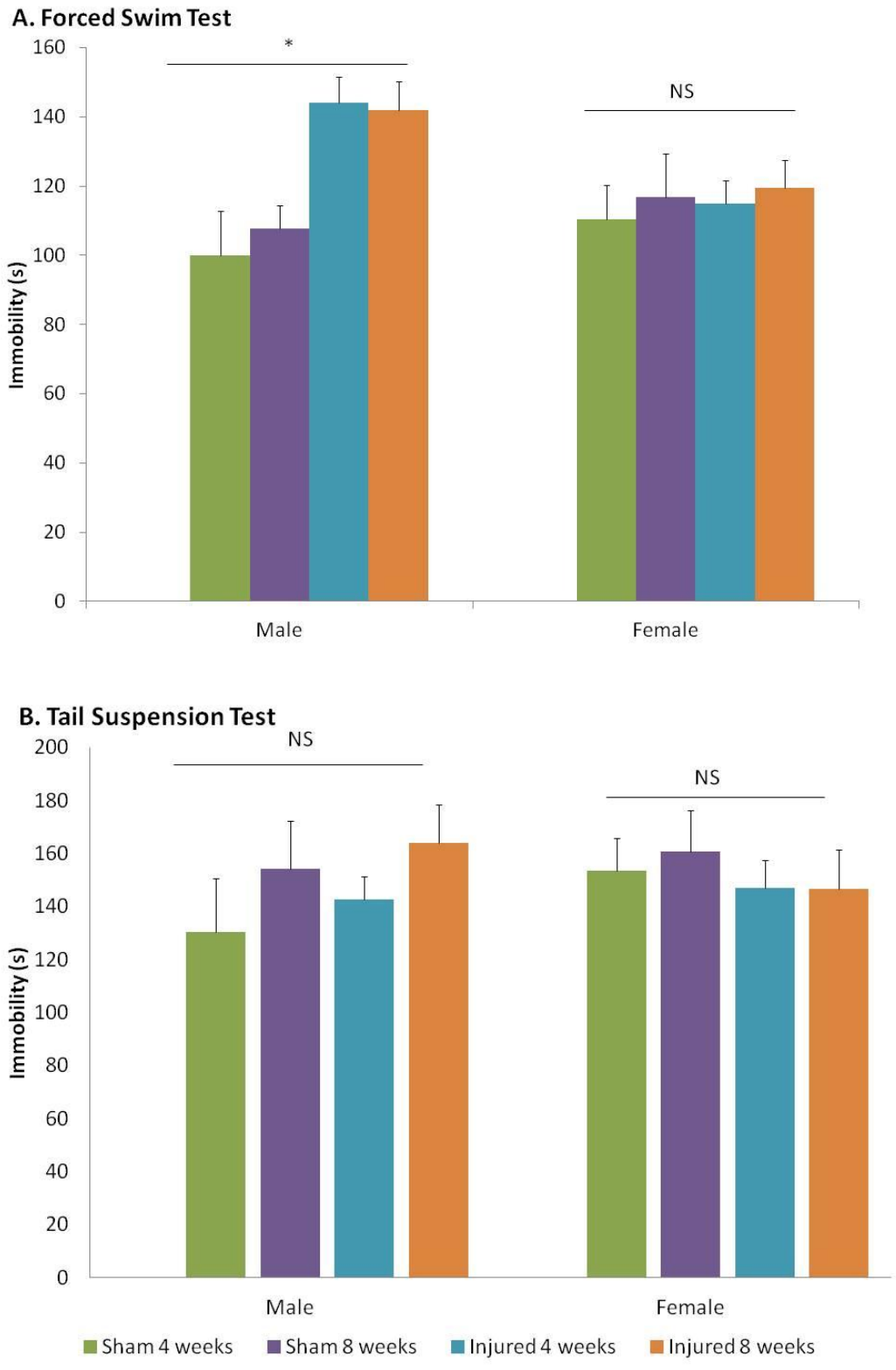

A. Forced Swim Test
*
NS
Immobility (s)
160
140
120
100
80
60
40
20
0
Male
Female
B. Tail Suspension Test
NS
NS
Immobility (s)
200
180
160
140
120
100
80
60
40
20
0
Male
Female
Sham 4 weeks
Sham 8 weeks
Injured 4 weeks
Injured 8 weeks

Figure 2.4

Figure 2.5: mTBI increases periorbital mechanical sensitivity in female brain-injured mice. Mice were tested for periorbital mechanical sensitivity using von Frey testing at 5 and 8 weeks post-injury. Repeated measures ANOVA indicated a significant difference in periorbital threshold (A) at both 5 and 8 weeks in female brain-injured mice compared to female sham injured mice ($p = 0.003$) while no significant difference was observed between male brain-injured and male sham injured mice ($p > 0.05$). Percent of animals developing an allodynic threshold is shown in (B). For male mice, the percent of animals with an allodynic threshold decreased between 5 and 8 weeks in sham injured mice and remained unchanged in brain-injured mice. There was no significant difference in number of brain-injured or sham injured male mice displaying allodynia at either time ($p > 0.05$). Percent of sham injured female mice displaying allodynia was unchanged between 5 and 8 weeks, and percent of brain-injured female mice displaying allodynia decreased from 100% to 71% of the group. A significant difference was observed between number of brain-injured and sham injured female mice displaying allodynia at 5 weeks ($p = 0.0038$) but not at 8 weeks ($p > 0.05$). Figures are pictured as group mean ± Standard Error of the Mean (SEM); *$p < 0.05$ compared to sham injured mice; NS = not significant.

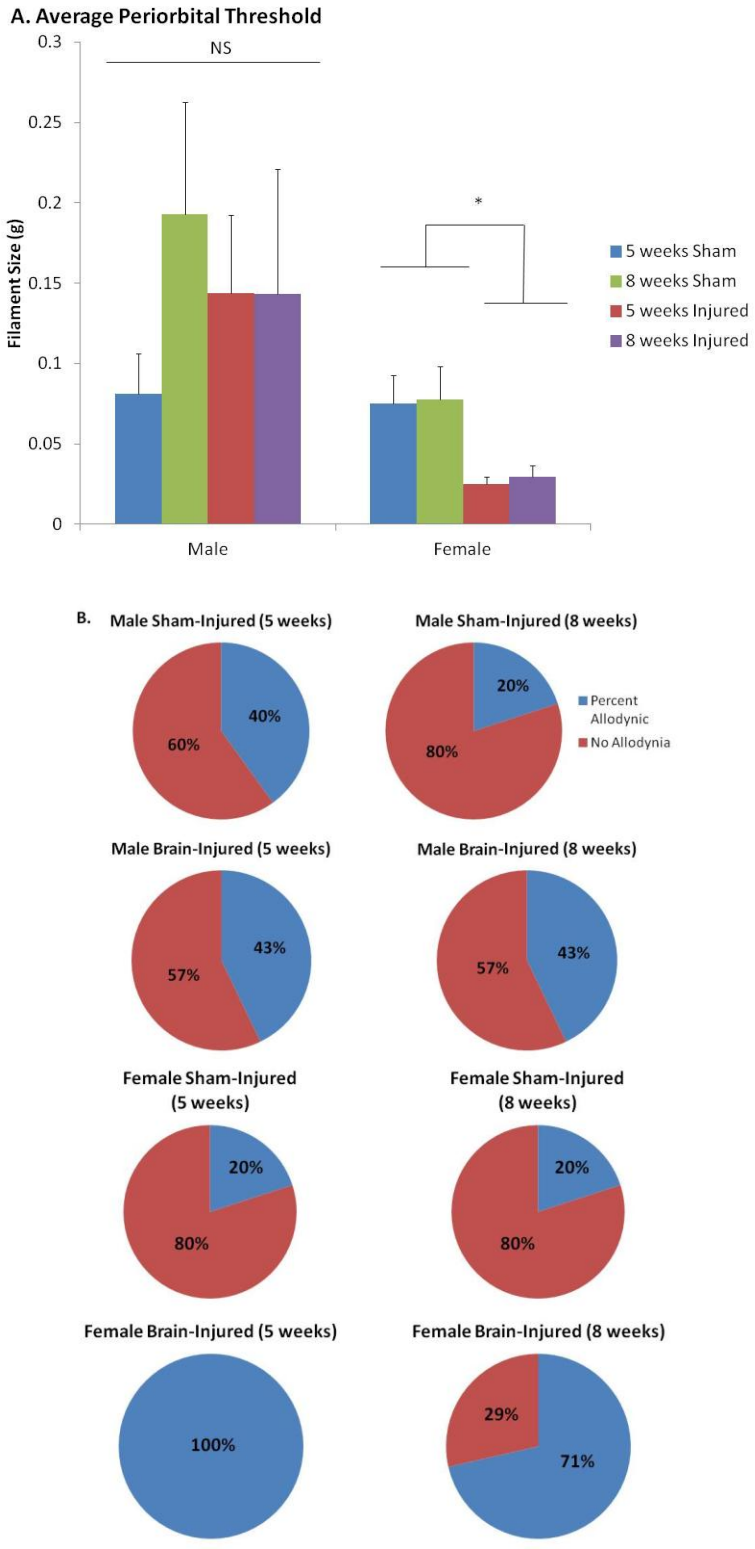

A. Average Periorbital Threshold
NS
*
Filament Size (g)
0.3
0.25
0.2
0.15
0.1
0.05
0
Male
Female
5 weeks Sham
8 weeks Sham
5 weeks Injured
8 weeks Injured
B.
Male Sham-Injured (5 weeks)
40%
60%
Male Sham-Injured (8 weeks)
20%
80%
Percent Allodynic
No Allodynia
Male Brain-Injured (5 weeks)
43%
57%
Male Brain-Injured (8 weeks)
43%
57%
Female Sham-Injured (5 weeks)
20%
80%
Female Sham-Injured (8 weeks)
20%
80%
Female Brain-Injured (5 weeks)
100%
Female Brain-Injured (8 weeks)
29%
71%

Figure 2.5

Figure 2.6: Distribution of periorbital thresholds in mice. Periorbital threshold for sham injured and brain-injured mice at 2 weeks and 4 weeks is given for male (A) and female (B) mice. Means are shown as horizontal lines for each group. There was no significant difference between thresholds of brain-injured and sham injured male mice at 5 weeks or at 8 weeks ($p > 0.05$). However, a Mann-Whitney U test indicated a significant difference between brain-injured and sham injured female mice at 5 weeks ($U = 2.5$, $p = 0.015$) but not at 8 weeks ($U = 6.5$, $p > 0.05$).

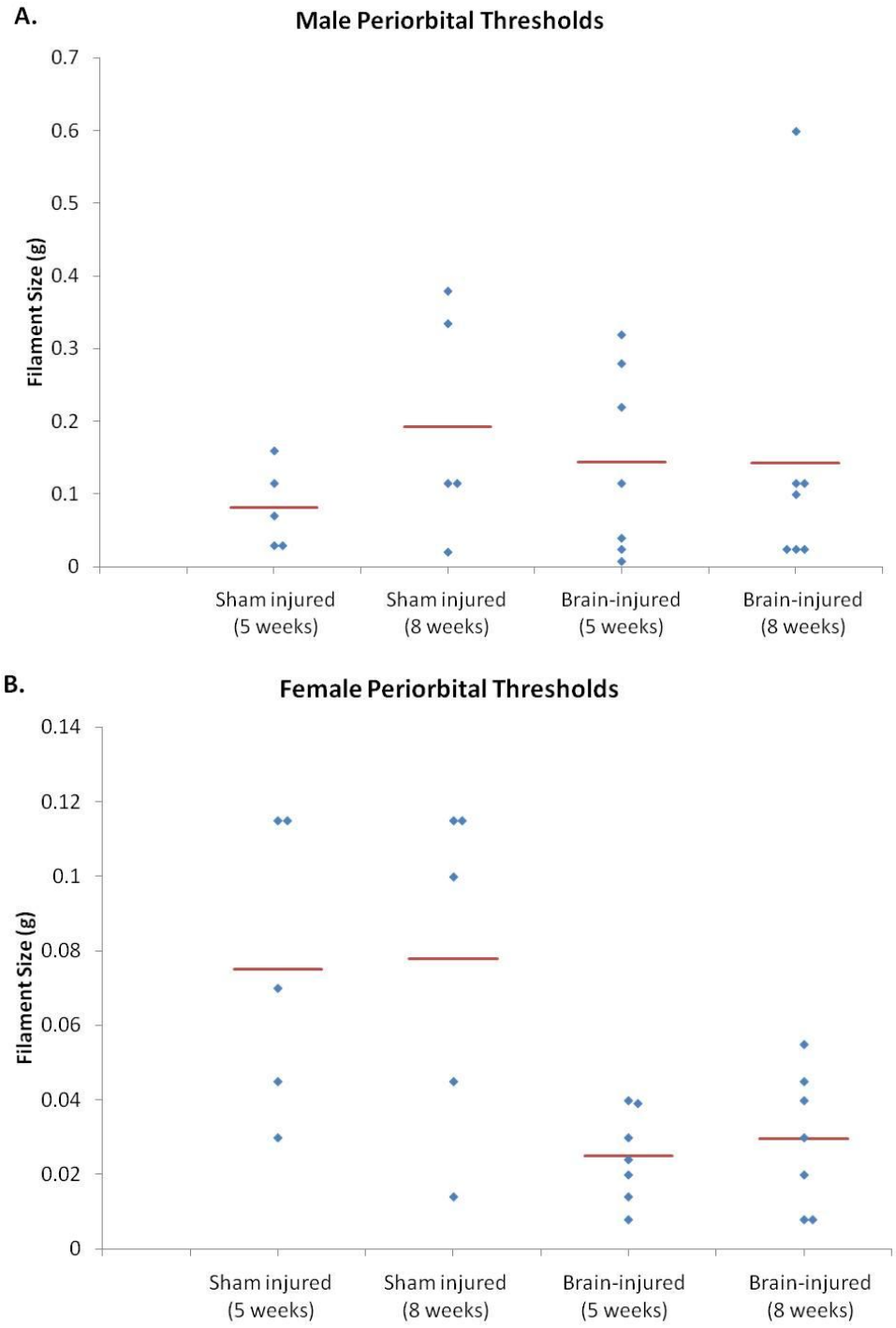

A.
Male Periorbital Thresholds
Filament Size (g)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Sham injured (5 weeks)
Sham injured (8 weeks)
Brain-injured (5 weeks)
Brain-injured (8 weeks)
B.
Female Periorbital Thresholds
Filament Size (g)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Sham injured (5 weeks)
Sham injured (8 weeks)
Brain-injured (5 weeks)
Brain-injured (8 weeks)

Figure 2.6

Figure 2.7: Representative voltammagrams of male mice. Representative traces are given for male sham injured mice before (A) and after (B) administration of cocaine and for male brain-injured mice before (C) and after (D) administration of cocaine. Stimulations begin at the bottom of the peak, lasting for 0.5 seconds (30 pulses at 60 Hz) and ending just before the peak release of dopamine (DA). Tau is representative of the reuptake of extracellular DA, shown as the portion of the trace extending from the peak to approximately a 60% return to pre-stimulation levels. Recordings last a total of 15 seconds. At baseline, peaks are similar between sham injured and brain-injured male mice while after administration of cocaine brain-injured male mice have a higher release of DA than sham injured mice.

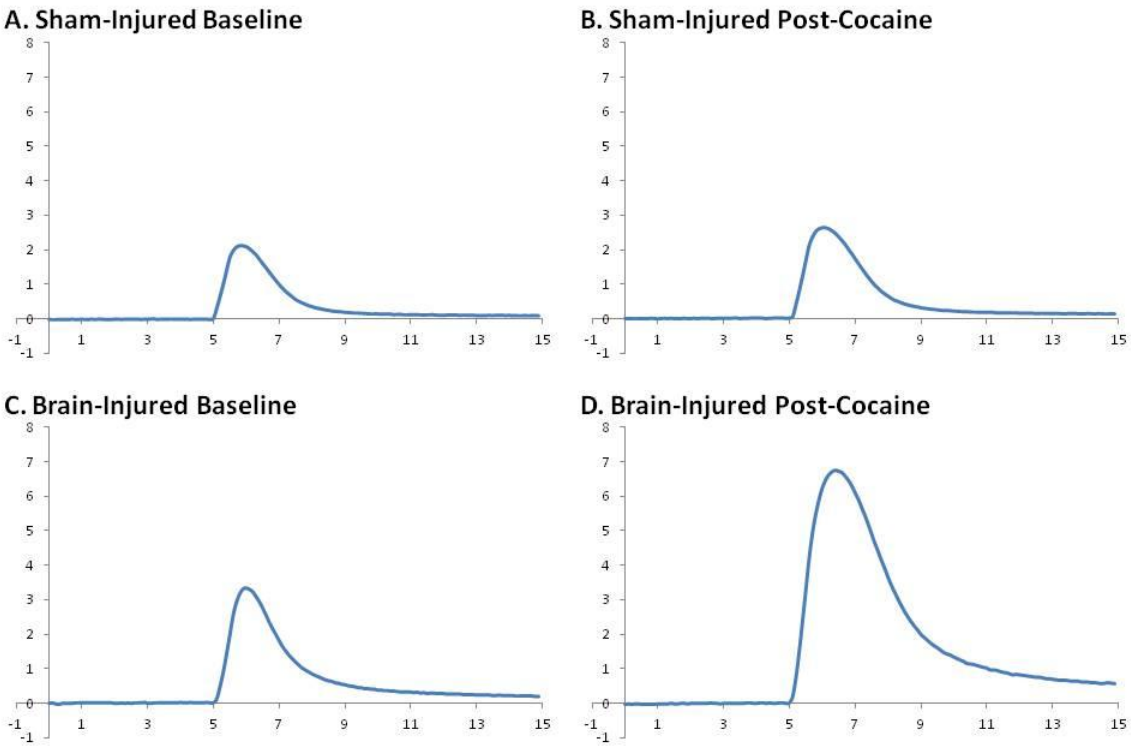

A. Sham-Injured Baseline
B. Sham-Injured Post-Cocaine
C. Brain-Injured Baseline
D. Brain-Injured Post-Cocaine

Figure 2.7

Figure 2.8: Representative voltammagrams of female mice. Representative traces are given for female sham injured mice before (A) and after (B) administration of cocaine and for female brain-injured mice before (C) and after (D) administration of cocaine. Stimulations begin at the bottom of the peak, lasting for 0.5 seconds (30 pulses at 60 Hz) and ending just before the peak release of dopamine (DA). Tau is representative of the reuptake of extracellular DA, shown as the portion of the trace extending from the peak to approximately a 60% return to pre-stimulation levels. Recordings last a total of 15 seconds. Post-cocaine peaks are higher than baseline in both sham injured and brain-injured female mice and while not significant, brain-injured baseline peaks are higher than sham injured baseline peaks.

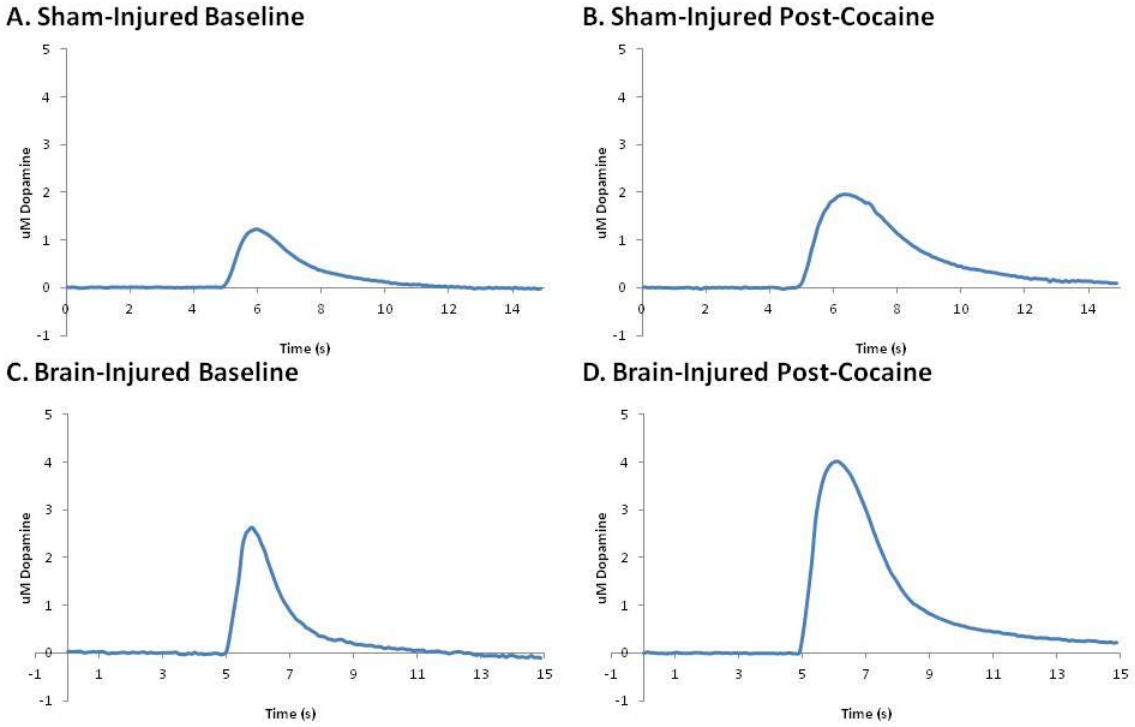

A. Sham-Injured Baseline
B. Sham-Injured Post-Cocaine
C. Brain-Injured Baseline
D. Brain-Injured Post-Cocaine
uM Dopamine
Time (s)

Figure 2.8

Figure 2.9: Extracellular dopamine concentration increases in male brain-injured mice following cocaine challenge. Amounts of extracellular dopamine (DA) were calculated before and after inhibition of the dopamine transporter (DAT) using cocaine. At baseline (A), two-way ANOVA revealed no significant differences in amounts of evoked DA release between male and female mice or between sham injured and brain-injured mice in either males or females ($p > 0.05$). Following administration of cocaine (B), a significant difference was observed between sham injured and brain-injured male mice ($p < 0.05$) and between brain-injured male and brain-injured female mice ($p < 0.05$). Figures are presented as group mean ± Standard Error of the Mean (SEM); *$p < 0.05$

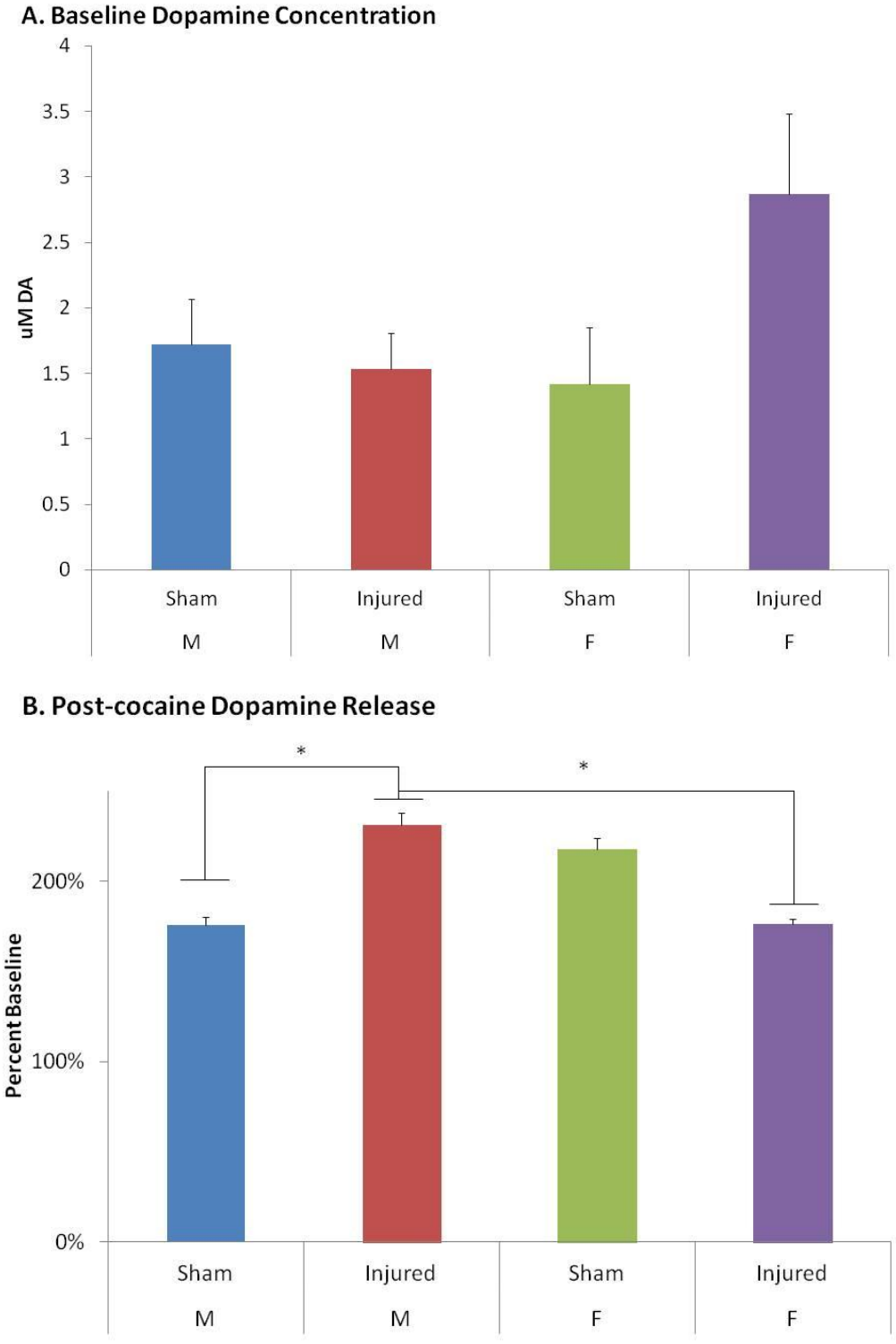

A. Baseline Dopamine Concentration
uM DA
4
3.5
3
2.5
2
1.5
1
0.5
0
Sham
M
Injured
M
Sham
F
Injured
F
B. Post-cocaine Dopamine Release
Percent Baseline
200%
100%
0%
*
*
Sham
M
Injured
M
Sham
F
Injured
F

Figure 2.9

Figure 2.10: There are no significant changes in *tau* at baseline or following cocaine challenge in either male or female mice. Differences in *tau* were calculated before and after inhibition of the dopamine transporter (DAT) using cocaine. At baseline (A) and following administration of cocaine (B), two-way ANOVA revealed no significant differences between male and female mice or between sham injured and brain-injured mice ($p > 0.05$). Figures are presented as group mean ± Standard Error of the Mean (SEM).

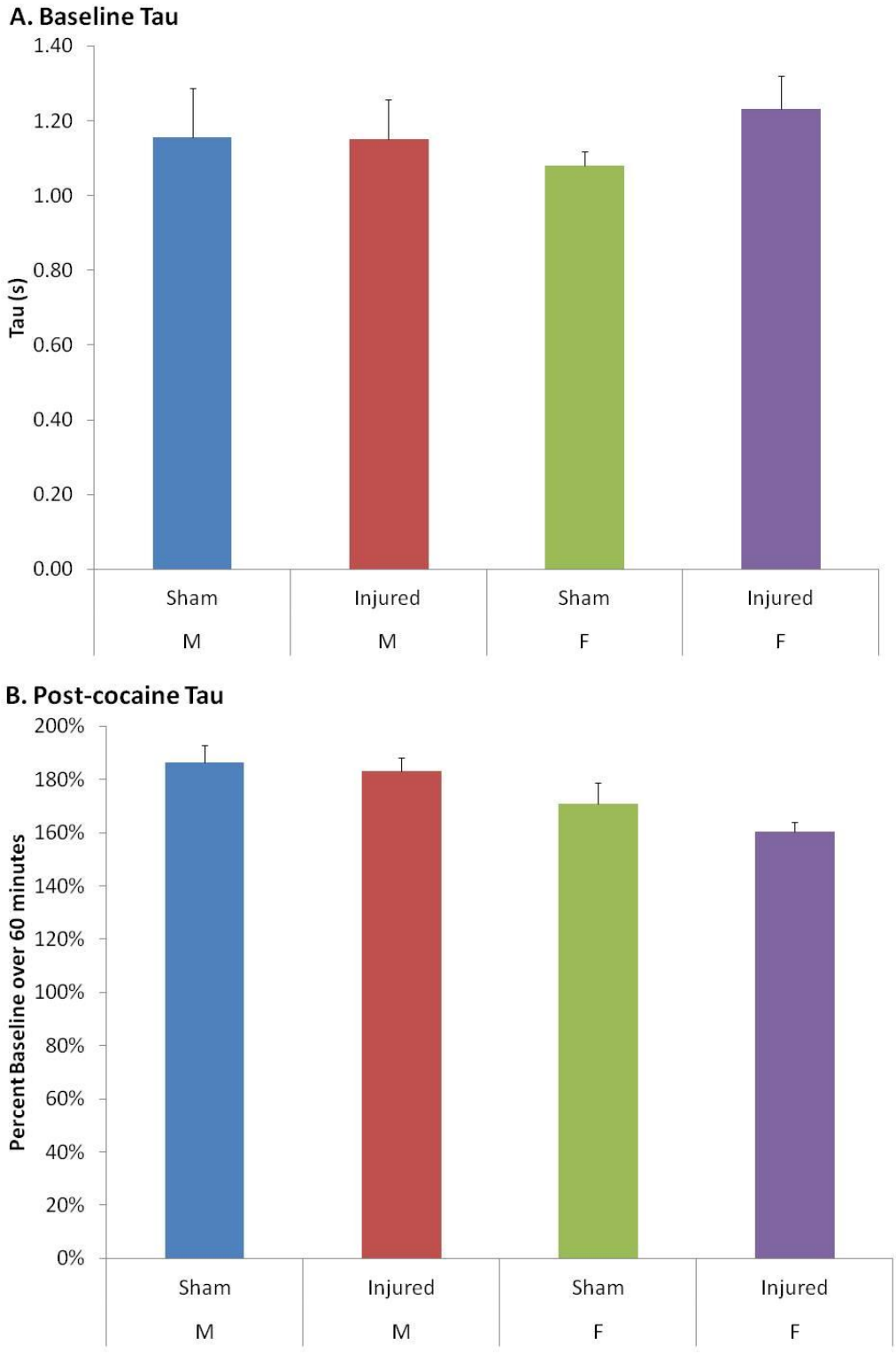

A. Baseline Tau
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Tau (s)
Sham
M
Injured
M
Sham
F
Injured
F
B. Post-cocaine Tau
200%
180%
160%
140%
120%
100%
80%
60%
40%
20%
0%
Percent Baseline over 60 minutes
Sham
M
Injured
M
Sham
F
Injured
F

Figure 2.10

CHAPTER THREE

INFLUENCES OF EXERCISE PRIOR TO REPEATED MILD TRAUMATIC BRAIN INJURY ON DEPRESSIVE-LIKE BEHAVIOR AND POST-TRAUMATIC PERIORBITAL ALLODYNIA

3.1 ABSTRACT

Athletes represent a population of people who are at risk for sustaining repetitive mild traumatic brain injuries (rmTBI) over the course of their playing career. A large number of studies have shown that exercise strengthens the brain, yet no studies utilize exercised animals, instead only using exercise as a therapeutic intervention following traumatic brain injury (TBI). Therefore, in this study I used a model of rmTBI in both exercised and unexercised mice to examine the effect of pre-injury exercise on development of depressive-like behavior and periorbital allodynia, models for post-traumatic depression and post-traumatic headache (PTH). I observed that rmTBI decreased time immobile in the forced swim test for depressive-like behavior and increased threshold of periorbital mechanical stimulation in brain injured male mice at 2 and 4 weeks following the final injury. Additionally, rmTBI decreased time immobile in brain-injured female mice compared to sham injured female mice, independent of exercise, at both 2 and 4 weeks post-injury, and periorbital threshold in unexercised female mice decreased at 4 weeks compared to 2 weeks. These results are contrary to previously published reports of rmTBI which do not include an exercise component, and this may be due to methodological issues such as repeated exposure to isoflurane or lack of sensitivity of testing methods. These results indicate that pre-injury exercise does not alter outcome following rmTBI in mice, but they demonstrate the sex-specific effects of rmTBI on development of both emotional and physical symptoms.

3.2 INTRODUCTION

3.2.1 Beneficial effects of exercise

The beneficial effects of exercise are often reported and include improvements in general health, psychological health, and cognitive function (Dishman et al., 2006). In studies of aging, exercise has been shown to improve short-term memory or prevent memory failure, improve object recognition, and improve spatial learning (van Praag et al., 1999, O'Callaghan et al., 2007, Kim et al., 2010b). Additionally, in animal models of development and aging, voluntary exercise has been shown to alter expression of a number of proteins involved in neuronal plasticity including the glucose transporter GLUT1, brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and NMDA receptor subunit 2B, in addition to increases in hippocampal neurogenesis (Neeper et al., 1996, Gomez-Pinilla et al., 2002, Farmer et al., 2004, van Praag et al., 2005, Dishman et al., 2006, Allen and Messier, 2013). Changes such as increased hippocampal neurogenesis and activity-dependent synaptic plasticity have been correlated with the reported improvements in learning and memory (van Praag et al., 2005, Vivar et al., 2013). Functionally, voluntary exercise in rodents has been shown to increase long term potentiation (LTP) as well as reduce the threshold at which LTP is induced compared to non-runners (Farmer et al., 2004). Increases in cell proliferation and expression of exercise-mediated proteins including BDNF as well as reductions in aging-induced apoptosis have also been observed using forced exercise paradigms such as forced running wheel or treadmill training (Dishman et al., 2006, Chae and Kim, 2009, Kim et al., 2010b, Llorens-Martin et al., 2010, Ma et al., 2012). In moderate treadmill training, a decrease in GFAP content, a marker for astrocyte reactivity, and an increase in

glutamine sythetase activity was observed in the hippocampus of running mice, a finding suggestive of neuroprotection (Bernardi et al., 2013). In addition to neuronal and glial cell changes, improvements in spatial learning and memory are also observed following forced exercise (Kim et al., 2010b).

3.2.2 Exercise in brain injury and disease

Exercise has been used in several different models of brain injury and disease prior to insult or disease course. A number of studies have examined the influence of exercise preconditioning on acute outcomes after an ischemic event. As little as 2 to 3 weeks of exercise preconditioning is sufficient to induce neuroprotective effects acutely after an ischemic event, including a reduction in the release of glutamate and increased GABA release post-ischemia, potentially protecting neurons from cytotoxic damage (Wang et al., 2001, Jia et al., 2009, Wang et al., 2014). Exercise preconditioning has also been shown to reduce infarct volume, improve energy metabolism, reduce neuronal cell death, improve astrocytic glutamate regulation, reduce expression of inflammatory mediators, improve blood brain barrier function, and reduce production of reactive oxygen species following an ischemic event in rodents (Liebelt et al., 2010, Yang et al., 2012, Dornbos et al., 2013, Ma et al., 2013, Wang et al., 2014).

While the majority of studies using exercise preconditioning show beneficial outcomes, a few studies in Huntington's disease models have shown exercise to worsen functional outcomes. In the Huntington's disease R6/2 mouse, voluntary running wheel exercise prior to symptom onset has been observed to accelerate symptom onset in male mice and worsen performance in behavioral and motor tasks including the Morris water maze and rotarod (Potter et al., 2010, Vivar et al., 2013). Additionally, the relationship

between sex or gender and exercise is a highly understudied aspect of exercise research despite evidence that exercise influences male and female animals differently. One study showed that in a model of voluntary running wheel, distance run correlated with alcohol consumption in female but not in male mice (Gallego et al., 2015). In addition, another study compared the effects of treadmill exercise in wild type and ALS transgenic male and female mice. They observed that exercise-induced increases in cell proliferation and BDNF mRNA were greater in male than in female mice (Ma et al., 2012). Finally, voluntary exercise in female rats has been shown to increase neuronal cell loss following kainate lesion in hippocampal CA2 and CA3 regions, but it is unclear if this effect is specific to female rats (Ramsden et al., 2003).

3.2.3 Exercise and stress

Exercise has been observed to be beneficial in attenuating detrimental responses to stress, one factor that can influence development of depression or depressive-like behavior. In one study, chronically exercised ovarectomized female mice displayed attenuated increases in depressive-like behavior following repeated restraint stress (Han et al., 2014). Another study showed that running in mice prevented stress-induced activation of new neurons in the ventral dentate gyrus and increased GABA release, offering protection in this model of stress as well (Schoenfeld et al., 2013).

3.2.4 Exercise and depression

A number of clinical studies have indicated that both acute and regular exercise can improve depressive symptoms as well as protect against development of depression and anxiety disorders and improve overall psychological well-being (Strohle, 2009). A single bout of exercise has been shown to acutely improve mood, regardless of age or gender,

but another study has shown this acute improvement only in regular exercisers (Dimeo et al., 2001, Bartholomew et al., 2005, Hoffman and Hoffman, 2007). Despite these contrary results, exercise has been shown to have a positive effect on several mood symptoms, particularly with regular moderate intensity exercise (Bartholomew et al., 2005). This includes improvements in general mood as well as symptoms of depression and anxiety, and this has been seen in both healthy controls and psychiatric patients (Hoffman and Hoffman, 2007). Furthermore, it has been shown that exercise as a treatment for depression is as effective as psychotherapy and antidepressant treatment (Hoffman and Hoffman, 2007). Exactly what causes this improvement in mood with exercise remains unclear due to little consistency in evaluation and lack of systemic studies, but exercise-induced expression of different growth factors such as vascular endothelial growth factor, associated with hippocampal neurogenesis which is down-regulated in depression, could explain some of the benefit observed in clinical populations (Fabel et al., 2003, Strohle, 2009).

3.2.5 Exercise and pain

Studies examining the influence of exercise on chronic pain in humans have given mixed results, a finding that is thought to be due to low quality of study design and lack of follow-ups in existing studies (Lawlor and Hopker, 2001, Meeus et al., 2010). In a study of chronic fatigue syndrome patients it was found that exercise decreased the threshold for pain whereas threshold was increased in control patients, indicating that in this condition exercise increases sensitivity to pain (Whiteside et al., 2004). Conversely it was found in chronic, but not acute, low back pain patients that exercise may be effective at reducing pain; another study showed no effect of exercise on back pain after 10 weeks,

but at a 2.5 year follow-up, exercised patients had fewer prescriptions for pain medications and physical therapy referrals, indicating that exercise may be beneficial for long-term outcome (Sculco et al., 2001, Hayden et al., 2005). Studies of fibromyalgia have shown mixed results, and one study examined the effects of different types of exercise on fibromyalgia pain, finding that pool-trained patients had greater improvements in self-reported physical impairment and pain compared to land-trained patients (Jentoft et al., 2001, Hoffman and Hoffman, 2007). In addition to studies in chronic pain patients, it has been reported that pain threshold is higher in athletes than in non-athletes, a finding that is consistent with studies in healthy individuals following acute bouts of exercise and exposure to several different painful stimuli (Hoffman and Hoffman, 2007). It has been suggested that beneficial effects of exercise on perception of pain relies on regular exercise of at least 70% maximal aerobic capacity, but this remains unclear due to inconsistencies in study design and may explain the varied results reported in clinical literature (Hoffman and Hoffman, 2007).

Some preclinical studies have also begun to examine the influence of exercise on pain perception, but these studies are limited. For example, it was found that following spinal cord injury (SCI) in rats acute exercise prevents onset of neuropathic pain and sprouting of c-fibers, possibly due to exercise-induced increases in neurotrophic factors, and this finding is consistent with earlier reports of exercise-induced analgesia in SCI pain (Hutchinson et al., 2004, Detloff et al., 2014).

3.2.6 Exercise and TBI

Preclinical TBI studies have mostly utilized exercise as a therapeutic intervention following injury rather than prior to TBI, and only male animals have been used.

Behaviorally, exercise has been shown to improve learning and memory following TBI in tasks including object recognition, step-down avoidance, and the radial 8-arm maze, and some studies report improvements in Morris water maze performance (O'Callaghan et al., 2007, Kim et al., 2010a, Vivar et al., 2013). A number of studies by Griesbach et al. have shown that exercise following TBI may be beneficial to functional recovery, but outcome is dependent on timing of exercise, intensity of exercise, and injury severity (Griesbach et al., 2004a, Griesbach et al., 2004b, Griesbach et al., 2007, Griesbach et al., 2009, Griesbach et al., 2012). Others have shown the involvement of exercise-upregulated pathways in brain repair, also supporting the idea that exercise is beneficial to recovery following TBI (Chen et al., 2012). In particular, one of these studies showed that voluntary exercise led to significant increases in hippocampal BDNF when exercise began 2 weeks after mTBI; however, in moderately injured animals, this effect was absent if exercise was initiated any earlier than 30 days post- injury (Griesbach et al., 2007). Another study showed that voluntary exercise but not forced exercise increased BDNF, possibly due to inhibiting effects of glucocorticoids from the stress inherent in forced exercise paradigms (Griesbach et al., 2012). At the cellular level, exercise following TBI has been shown to reduce apoptosis, reduce Purkinje cell loss in cerebellum, reduce astrocyte reactivity and GFAP content in cerebellum and hippocampus, increase BDNF expression, and increase markers for cell proliferation including nestin and Ki67 (Griesbach et al., 2004a, 2007, Kim et al., 2010a, Seo et al., 2010, Itoh et al., 2011). However, the majority of these studies use voluntary exercise, post-injury exercise alone, and only male animals, leaving several key questions to be answered.

3.2.7 Significance

Several studies have developed animal models of rmTBI as a model of athlete injury, but none of these models accounts for the effects of exercise on outcomes following injury or the response of the brain to repeated impacts. It has been demonstrated in a number of clinical and preclinical studies that exercise alters the brain at the cellular and functional level, leading to improvements in cognitive function and development of depressive behavior as a result of stress. Studies which examine exercise following TBI have shown that exercise can improve functional deficits in cognition as well as death and damage of cells in a manner dependent on injury severity, timing after injury, and exercise paradigm. However, no studies in TBI have examined the influence of exercise prior to injury on outcome, and no rmTBI models include exercise; furthermore, the majority of these studies utilize only male animals, and clinical studies fail to separate men and women into separate groups despite evidence for gender-specific outcomes. In order to create a more relevant model of rmTBI in athletes, I used a paradigm of pre-injury exercise in both male and female mice and evaluated functional outcomes of post-traumatic pain and depressive behavior.

3.3 MATERIALS AND METHODS

3.3.1 Animals

Male and female C57BL/6J mice (aged 6-8 weeks; Jackson Laboratories, Bar Harbor, ME) were used for this study. Animals received standard chow *ad libitum* and were maintained four mice to a cage in a controlled temperature environment with a 12 hour light-dark cycle. All procedures used followed guidelines set by the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and these procedures were approved by the Drexel University Institutional Animal Care and Use Committee.

3.3.2 Treadmill Exercise

Treadmill exercise was conducted for 15 days as previously published (Liu et al., 2009, Um et al., 2011, Nam et al., 2014, Tuon et al., 2014). Briefly, mice were acclimated to a motorized treadmill (Columbus Instruments, Columbus, OH) for 3 days at 5 m/min for 10 minutes per day; acclimation times were to train mice to run on the treadmill and reduce stress. Tail touch was used for the duration of exercise to motivate mice to run; no shock was used. Following acclimation, mice ran on the treadmill at a moderate intensity for 15 consecutive days. On the first day after acclimation, mice ran for a total of 20 minutes while the speed was increased from 5 to 10 m/min. For the next four days, speed was maintained at 10 m/min while duration was gradually increased up to 60 minutes. For the final 10 days, speed was maintained at 10 m/min and duration was constant at 60 minutes per day. The day after exercise ended, animals underwent concussive brain injury surgery.

A separate set of mice was used for evaluation of cell proliferation changes in order to confirm efficacy of moderate treadmill training in our facility. Mice were acclimated for 3 days followed by 10 days of exercise over a 2 week period and injected with 50 mg/kg bromodeoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO) on days 5 through 10 of exercise. These mice were sacrificed at the conclusion of exercise on the final day of training.

3.3.3 Repeated Concussive Brain Injury

Mice received three mild closed head impacts with a 48 hour inter-impact interval using the same methods as previously published to produce a single mild closed head injury (Creed et al., 2011). Mice were anesthetized using isoflurane (3%; Penn Veterinary Supply, Lancaster, PA) inhalation via a nose cone. Body temperature was maintained during surgery using a heating pad set to 37°. The scalp was swabbed with povidone-iodine and isopropyl alcohol, and mice received a subcutaneous injection of lidocaine prior to making a 1.0 cm midline rostral to caudal incision in the scalp. The periosteum was reflected and mice were placed in a standard mouse restrainer (Braintree Scientific, Braintree, MA) with the head supported by a soft foam pad so that it was level with the body. The mouse in the restrainer was then positioned beneath the cortical impact device (Custom Design & Fabrication, Richmond, VA), and a 5-mm-diameter hemispheric metal impactor tip was zeroed against the exposed skull by touching it to the sagittal suture midway between the bregma and lambda sutures. Thirty seconds after removal of isoflurane, the impactor tip was electronically driven perpendicularly onto the exposed sagittal suture at a velocity of 5.0 m/s and a depth of 1.5 mm beyond the zero point. Immediately following injury, righting reflex was obtained by evaluating the amount of

time until the mouse regained normal posture over three consecutive attempts when placed in a supine position. Following righting reflex, animals were re-anesthetized and scalp incisions sutured with 4-0 silk sutures. Sham-injured mice received the same surgical procedures without receiving an impact from the metal impactor tip. All animals were allowed to recover on a heating pad set at 37° and were returned to home cages once they became sternally recumbent. All mice received injections of Buprenex (0.05 mg/kg; McKesson, San Francisco, CA) following injury and at 12 hours post-surgery. Surgical procedures were repeated at 48 intervals for a total of three impacts or three surgeries and occurred during the afternoon and evening with individual animals receiving each impact at approximately the same time of day for successive surgery days.

3.3.4 Assessment for Depressive-like Behavior

In order to test for depressive-like behavior, the forced swim test (FST) was used, and animals were evaluated at 2 and 4 weeks post-injury or post-surgery. In the FST, mice are placed in a beaker of water that is deep enough that the animal cannot rest its tail on the bottom of the beaker but not filled so high that the mouse can jump out. Water temperature is kept between 23 and 25°C to reduce variability of behavior (Petit-Demouliere et al., 2005). Animals are recorded for 6 minutes, and time immobile, when the mouse is not actively swimming, is measured. Paddling without movement is considered immobility, as it has been identified as the least amount of effort necessary for the mouse to remain afloat (Petit-Demouliere et al., 2005). Time immobile is considered to be a readout for depressive-like behavior, or “despair,” and immobility is measured for the last 4 out of 6 minutes of the test.

3.3.5 Assessment for Mechanical Sensitivity

In order to test for changes in mechanical stimulation and development of facial allodynia following injury, I used the ascending method of von Frey (VF) stimulation, as previously published (Elliott et al., 2012, Macolino et al., 2014). Animals were evaluated at 4 and 8 weeks post-injury or post-surgery. Mice were acclimated to the testing room for one hour before being placed in a standard plastic restrainer (Braintree Scientific, Braintree, MA) at a 30° angle for a maximum of 15 minutes. Von Frey filaments of sizes 0.008, 0.02, 0.04, 0.07, and 0.16 g were applied to the periorbital area 5 times bilaterally with 10 seconds between stimulations for each filament beginning with the smallest filament and progressing to larger filaments until the threshold was obtained (Elliott et al., 2012). Positive responses were recorded as head shaking, stroking the face with the forepaw, or withdrawal from stimulus, and thresholds were considered to be 3 or more positive responses out of 5 on each side, or at least 60% positive responses for the filament. All facial allodynia testing occurred before noon on the day of testing to reduce variability. Allodynic thresholds were determined as values at or below 0.04 g, or two filament sizes below 0.1 g.

3.3.6 Statistical Analyses

Quantifications were performed with experimenters who were blinded to injury status of the mice. Data are expressed as mean $\pm$ standard error of the mean (SEM). Comparisons of righting reflex times, immobility times for FST, and threshold values for VF testing were determined using repeated measures analysis of variance (ANOVA) followed by Newman-Keuls *post-hoc* if ANOVA rejected the null hypothesis. Significance for percent allodynia was determined using Chi Square analysis. Distributions of periorbital

thresholds were evaluated using the Kruskal-Wallis one way ANOVA followed by a Mann-Whitney U test if ANOVA rejected the null hypothesis. Only p values < 0.05 were considered to be statistically significant.

3.4 RESULTS

3.4.1 Effects of exercise on cell proliferation

A separate group of mice was exercised and treated with BrdU to evaluate exercise-induced cell proliferation. Exercise induced an increase in BrdU-labeled cells in the cortex and subgranular zone of the hippocampus in both male and female mice (Figure 3.1). However, due to low subject number, no statistics were used to compare increases in proliferation.

3.4.2 Acute neurological responses after CCI

Impact with the metal impactor tip resulted in a minor fracture perpendicular to the sagittal suture after each injury in all but two of the injured and unexercised male mice; fractures occurred following the second and third injuries in these two mice. Fractures occurred following all three injuries in all injured and exercised male mice and in 10 out of 12 injured and unexercised female mice with the fractures occurring following the second and third impacts in the remaining two mice. Fractures occurred in all but one injured and exercised female mice following every injury; fractures occurred after the second and third impacts in the final mouse.

Of thirteen injured and unexercised male mice, two died immediately following the first injury and one died following the second injury. No mortality either acute or delayed occurred in injured and exercised male mice or injured and unexercised female mice. One of twelve injured and exercised female mice died approximately one hour after the third impact.

Sham injured mice spontaneously righted themselves approximately two to three minutes after removal of anesthesia during the first surgery, one to two minutes after

removal of anesthesia during the second surgery, and 30 to 90 seconds after removal of anesthesia during the third surgery. Brain-injured mice regained the righting reflex after a significantly longer duration following impact and removal of anesthesia compared to sham injured mice in both male and female mice, and the time to regain righting reflex decreased with each successive injury. In male mice (Figure 3.2), a repeated measures ANOVA indicated a significant effect of injury [$F(1,40) = 332.48$, $p < 0.001$], an interaction between exercise status and injury status [$F(1,40) = 7.27$, $p = 0.01$], an effect of number of injuries [$F(2,80) = 112.82$, $p < 0.001$], and an interaction between injury status and number of injuries [$F(2,80) = 36.25$, $p < 0.001$]. In female mice (Figure 3.3), repeated measures ANOVA indicated a significant effect of injury [$F(1,42) = 474.99$, $p < 0.001$], an effect of number of injuries [$F(2,84) = 111.77$, $p < 0.001$], and an interaction of number of injuries and injury status [$F(2,84) = 31.68$, $p < 0.001$]. Some brain injured male and female mice experienced a brief period of apnea lasting up to 12 seconds after the impact; no apnea was present in sham injured male or female mice.

3.4.3 Effects of exercise on depressive-like behavior

Mice were evaluated for depressive-like behavior using the FST. Repeated measures ANOVA indicated that all brain-injured male mice, independent of exercise status, had significantly less immobility compared to sham injured male mice at 2 and 4 weeks post-injury [$F(1,40) = 10.34$, $p = 0.003$] (Figure 3.4 A). Similarly, all brain-injured female mice, independent of exercise, had significantly less immobility than sham injured female mice at 2 and 4 weeks post-injury [$F(1,41) = 4.305$, $p = 0.04$] (Figure 3.4 B). No effect was seen between exercised and unexercised mice, independent of injury status, or between time points.

3.4.4 Effect of exercise preconditioning on threshold to mechanical stimulation

Mice were evaluated for periorbital mechanical sensitivity at 2 and 4 weeks post-injury. Thresholds for mechanical sensitivity in brain-injured male and female mice did not differ from sham injured mice, and exercise did not significantly affect threshold. However, repeated measures ANOVA indicated that male brain-injured mice had a significantly higher threshold for mechanical stimulation at 4 weeks than at 2 weeks, independent of exercise status [$F(1,40) = 9.09$, $p = 0.004$] and that all male mice, irrespective of injury or exercise status, had a significantly increased threshold at 4 weeks than at 2 weeks [$F(1,40) = 4.09$, $p < 0.05$] (Figure 3.5 A). Allodynic thresholds were defined as thresholds at or below 0.04 g, or two filament sizes below 0.1 g. At 2 weeks, 60% of unexercised brain-injured male mice display allodynia compared to 18% of unexercised sham injured male mice, and 50% of exercised brain-injured male mice display allodynia compared to 9% of exercised sham injured male mice. At 4 weeks, 30% of unexercised brain-injured male mice display allodynia compared to 27% of unexercised sham injured male mice while 25% of exercised brain-injured male mice display allodynia compared to 0% of exercised sham injured male mice (Figure 3.5 B). A Chi Square analysis indicated that there was a significant difference at 2 weeks ($X^2 = 8.64$, $n = 44$, $p = 0.035$) between unexercised brain-injured and sham injured male mice ($X^2 = 3.88$, $n = 21$, $p = 0.049$) and between exercised brain-injured and sham injured male mice ($X^2 = 4.54$, $n = 23$, $p = 0.033$), but there was no significance at 4 weeks ($X^2 = 3.86$, $n = 44$, $p > 0.05$). Distributions of periorbital thresholds are given for male mice in Figure 3.6. A Kruskal-Wallis one-way ANOVA indicated a significant difference between brain-injured and sham injured male mice ($H(7) = 14.82$, $p = 0.038$). A Mann-

Whitney U test for male mice at 2 weeks (Figure 3.6 A) indicated a significant difference between exercised sham injured and brain-injured male mice (U = 32.5, p = 0.036) but not between any other groups. No significant difference was observed at 4 weeks (Figure 3.6 B). Mann-Whitney U values are presented in Figure 3.6 C. Repeated measures ANOVA in female mice indicated an interaction effect of exercise status and time; unexercised female mice had a significantly lower threshold at 4 weeks than at 2 weeks [F (1,41) = 5.77, p = 0.02] (Figure 3.7 A). Injury did not affect threshold in female mice. At 2 weeks, 42% of unexercised brain-injured female mice developed allodynia compared to 25% of unexercised sham injured female mice, and 36% of exercised female brain-injured mice display allodynia compared to 60% of exercised sham injured mice. At 4 weeks, 58% of unexercised brain-injured female mice display allodynia compared to 50% of unexercised sham injured female mice, and 45% of exercised brain-injured female mice display allodynia compared to 40% of exercised sham injured female mice (Figure 3.7 B). A Chi Square analysis indicated no significant differences at either 2 weeks (X^2 = 4.37, n = 45, p > 0.05) or at 4 weeks (X^2 = 0.80, n = 45, p > 0.05). Distributions of periorbital thresholds are given for female mice in Figure 3.8. A Kruskal-Wallis ANOVA indicated no significant difference between brain-injured and sham injured female mice (H(7) = 5.07, p > 0.05). Mann-Whitney U values are presented in Figure 3.8 C.

3.5 DISCUSSION

In Chapter 2, I reported that following a single mild traumatic brain injury (mTBI), male brain-injured mice developed depressive-like behavior at 4 and 8 weeks post-injury as evaluated using the FST but not the TST, and female brain-injured mice developed decreased periorbital thresholds to mechanical stimulation at 5 and 8 weeks post-injury. Here, I observed that rmTBI decreased depressive-like behavior in both male and female mice compared to sham injured mice with no effect of exercise. Additionally, I observed a decrease in mechanical sensitivity in male mice that was independent of injury status and a decrease in sensitivity in brain-injured male mice at 4 weeks compared to 2 weeks post-injury. Finally, I observed an increase in mechanical sensitivity at 4 weeks compared to 2 weeks in unexercised female mice.

There have been increasing reports of development of depression and suicidality among retired professional athletes at a rate higher than the general population, particularly among athletes with a history of repeated concussion (Guskiewicz et al., 2007, Didehbani et al., 2013). These athletes demonstrate behavioral and personality changes including depression, apathy, irritability, and suicidality, and the brains of these athletes show evidence of chronic traumatic encephalopathy after death (Didehbani et al., 2013). Despite the expectation of exacerbated depressive-like behavior in my exercised mice, I observed that brain-injured mice of both sexes exhibited significantly less depressive behavior than sham injured mice of the same sex regardless of exercise. Additionally, this is in contrast to my earlier results showing that a single mTBI results in increased depressive-like behavior in male mice. It is possible that repeated exposure to isoflurane anesthesia altered the behavior of my mice or the effects of injury, particularly

since even the unexercised mice did not develop depressive behavior, in contrast to a previous report (Petraglia et al., 2014). It has been demonstrated that repeated isoflurane exposure can not only have facilitating effects on behaviors such as spatial memory and learning tasks but that it may also be protective against ischemic injury (Kapinya et al., 2002, Butterfield et al., 2004, Rammes et al., 2009). To determine if isoflurane is affecting outcome of these animals, a comparison to other anesthetic agents may be performed. In addition to my results occurring due to repeated isoflurane exposure, it is also possible that these mice developed hyperactivity as a result of repeated injury, masking depressive behavior. An increase in locomotor hyperactivity has previously been reported in a mouse model of rmTBI as well as other closed head injury models in mice, and this is also consistent with clinical studies showing that TBI may induce increased hyperactivity associated with inhibitory control disorders (Konrad et al., 2000, Pullela et al., 2006, Homsi et al., 2010, Kane et al., 2012). In fact, agitation syndromes including disinhibition and aggression symptoms have been reported in 5-71% of mTBI patients, regardless of gender (Kim, 2002, Kadyan et al., 2004). The decrease in immobility observed in my brain-injured male and female mice could be explained as agitation, fitting with the clinical and preclinical data regarding locomotor activity and disinhibition. Additionally, it is thought that loss of tonic balance in inhibitory circuits of the orbitofrontal and ventromedial coretex as well as disruptions in connections between cortical and limbic subcortical structures and increases in DA in the VTA may be related to these symptoms in animal models (Kim, 2002, Voon et al., 2010). As I previously observed altered DA signaling in male brain-injured mice following a single mTBI, it is possible that rmTBI in this model increased DA signaling or DA concentrations in these

regions, leading to increased locomotor activity that could indicate agitation syndromes including disinhibition or impulsivity. To address this possibility, evaluations of locomotor activity may be performed using tasks such as rotarod or the open field test. DA changes may also be evaluated using techniques such as microdialysis, fast scan cyclic voltammetry, or immunohistochemistry.

I also evaluated changes in periorbital mechanical sensitivity, a test for headache in rodents and a model for PTH in mice. In male mice, I observed a difference in the number of exercised brain-injured and sham injured mice displaying allodynia at 2 weeks but not between other groups or at 4 weeks. However, I observed an increase in periorbital threshold, indicating a decrease in sensitivity, that was independent of injury status as well as an increase in threshold at 4 weeks compared to 2 weeks in brain-injured male mice, and these changes were independent of exercise status. This may indicate that male brain-injured mice become hyposensitive with increasing number of injuries rather than developing allodynia. While I expected to see increased sensitivity, hyposensitivity could also represent a deficit in mechanical sensation following rmTBI. Hyposensitivty has the potential to lead to significant impairment in daily functioning as well as increased risk for serious bodily harm, as demonstrated by disorders of congenital insensitivity to pain (Golshani et al., 2014). While allodynia is thought to occur due to overactivation or rearrangement of nerve fibers following nerve injury, it is possible that a loss of fibers, similar to what has been suggested in congenital insensitivity to pain, may also lead to the hyposensitivity observed here (Sasnur et al., 2011, Detloff et al., 2014). Repeated mTBI has been shown to produce increased astrocyte reactivity, neuroinflammation, and neuron damage and dysfunction, suggesting that it may lead to

death and degeneration of axons that could contribute to pain neurotransmission (Longhi et al., 2005, Bolton and Saatman, 2014, Luo et al., 2014). Additionally, it has been reported that increasing DA within the mesocorticolimbic system may lead to analgesia, suggesting that similar to the decreased immobility observed in the FST, brain-injured male mice have increased DA activity following repeated injury (Sotres-Bayon et al., 2001, Finan and Smith, 2013). However, there is little clinical or preclinical research regarding pain hyposensitivity or insensitivity in TBI or other injury models, suggesting that this symptom has either not been previously observed or has not been detected. Finally, it is possible that repeated isoflurane exposure could also have influenced development of chronic central pain in my animals, but this is uncertain due to a lack of research regarding the effects of isoflurane on sensations of pain in rodents. In female mice, threshold decreased at 4 weeks compared to 2 weeks in unexercised mice, and while not significant, exercised female mice exhibited lower thresholds than unexercised mice at 2 weeks, levels that were maintained at 4 weeks. It is possible that I was unable to detect differences in periorbital threshold between sham injured and brain-injured females due to limitations in the sensitivity of the test. The von Frey monofilaments used in the test have varying amounts of force between consecutive filaments, some with increases of 0.012 g of force from one filament to the next but others with 0.24 g between consecutive filaments. The lack of intermediate sizes may account for my inability to detect differences between sham injured and brain-injured female mice. Additionally, periorbital von Frey testing has not been reported in female mice following any level of TBI. It is possible that a different test for evoked pain may be more sensitive to changes in periorbital sensitivity than von Frey testing in these female mice. These tests may

include pin prick, air puff, or cold sensitivity testing, all tests which have previously been used to test male animals in rodent models of headache and migraine disorders (Vos et al., 1994, Jeon et al., 2012, Dieb and Hafidi, 2013). Finally, I observed trend of a greater percentage of brain-injured mice displaying allodynia compared to sham injured mice in all groups except exercised female mice at 2 weeks. It is possible that exercise is protective acutely in these animals but that the protective effect is lost over time. However, it is unclear whether the loss of this trend is due to cessation of exercise following injury or whether exercise in female mice is neuroprotective acutely but not chronically. Additionally, there have been no previous studies examining periorbital allodynia in female mice, so it is possible that the value of 0.04 g that I used here is not appropriate for female mice; instead, since sham injured female mice display lower thresholds than their male counterparts, the threshold for allodynia may actually be lower. However, evaluation of naïve mice is necessary to understand how baseline thresholds may differ between male and female mice. I also observed that in my male mice, exercise reduced the number of animals that developed periorbital allodynia compared to unexercised male mice. This may suggest that exercise may have differing effects on development of periorbital allodynia between male and female mice.

In summary, I observed here a decrease in depressive-like behavior in both male and female brain-injured mice compared to sham injured mice as well as sex-specific changes in periorbital sensitivity. These results are contrary to previously published results as well as my previously reported changes in depressive-like behavior and periorbital sensitivity in mice following a single mTBI. The differences here may be due to methodological issues such as repeated isoflurane exposure or limited sensitivity of

utilized tests. However, additional behavioral tests such as the open field test or alternative tests for facial sensitivity may elucidate the differences in exercised and non-exercised sham injured and brain-injured mice. Despite the problems described here, this study demonstrates the sex-specific effects of rmTBI on development of emotional and physical symptoms of depression and PTH. Additionally, it lays the groundwork for future studies involving modifications to exercise paradigms around the time of injury, building a more relevant model of athlete TBI in mice.

3.6 FIGURES

Figure 3.1: Exercise increases cell proliferation in the hippocampus and cortex of male and female mice. A separate set of mice was used to test the moderate treadmill paradigm used in this study. We found that 10 days of moderate treadmill training was sufficient to increase cell proliferation in the cortex (CTX) and subgranular zone (SGZ) of the hippocampus in both male and female mice. Representative images are pictured in (A) and quantifications are given in (B). (n = 2-3 animals per group)

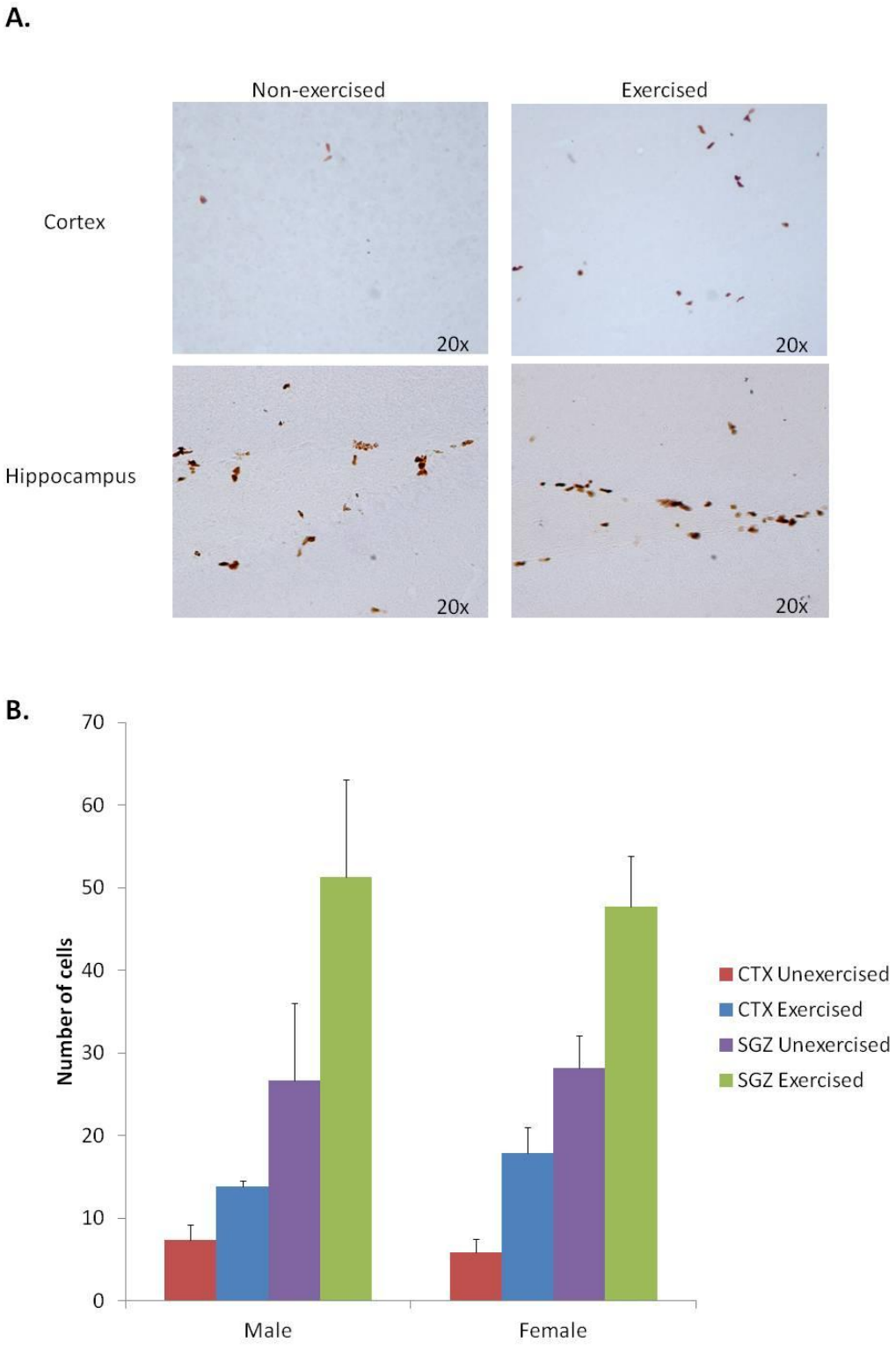

A.
Non-exercised
Exercised
Cortex
Hippocampus
20x
20x
20x
20x
B.
Number of cells
70
60
50
40
30
20
10
0
Male
Female
CTX Unexercised
CTX Exercised
SGZ Unexercised
SGZ Exercised

Figure 3.1

Figure 3.2: Righting reflex time increases after injury in male brain-injured mice. Righting reflex (RR) times for brain-injured male mice were significantly longer than RR times for sham injured mice after the first injury (RR1), second injury (RR2), and third injury (RR3) and for overall RR time across all three surgeries in both unexercised (A) and exercised (B) mice. Additionally, RR times were longer during the first surgery than the second and third surgeries in sham injured mice but there was no significant difference in RR time between the second and third surgeries. There was a significant difference between all RR times among brain-injured male mice. Finally, there was no difference between sham injured exercised and unexercised mice, but there was a significant increase in RR time in exercised brain-injured male mice compared to unexercised brain-injured male mice ($p = 0.002$). Figures are presented as group mean ± Standard Error of the Mean (SEM); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, N.S. = not significant.

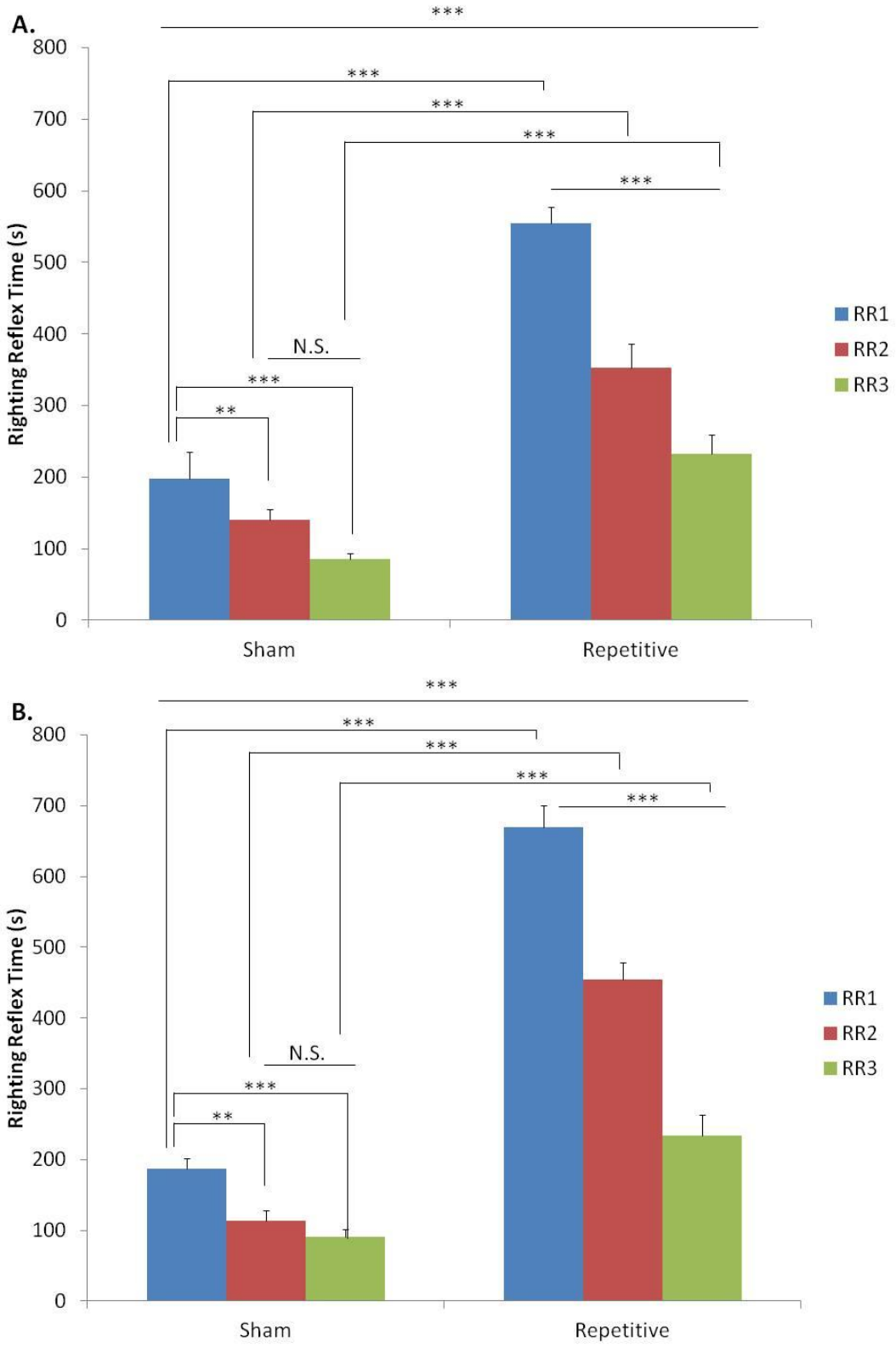

A.
***
***
***
***
***
N.S.
***
**
800
700
600
500
400
300
200
100
0
Righting Reflex Time (s)
Sham
Repetitive
RR1
RR2
RR3
B.
***
***
***
***
***
N.S.
***
**
800
700
600
500
400
300
200
100
0
Righting Reflex Time (s)
Sham
Repetitive
RR1
RR2
RR3

Figure 3.2

Figure 3.3: Righting reflex time increases after injury in female brain-injured mice. Righting reflex (RR) times for brain-injured female mice were significantly longer than RR times for sham injured mice after the first injury (RR1), second injury (RR2), and third injury (RR3) and for overall RR time across all three surgeries in both unexercised (A) and exercised (B) mice. Additionally, RR times were longer during the first surgery than the second and third surgeries in sham injured mice but there was no significant difference in RR time between the second and third surgeries. There was a significant difference between all RR times among brain-injured female mice. Finally, there was no difference between sham injured exercised and unexercised mice or between brain-injured exercised and unexercised mice. Figures are presented as group mean ± Standard Error of the Mean (SEM); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, N.S. = not significant.

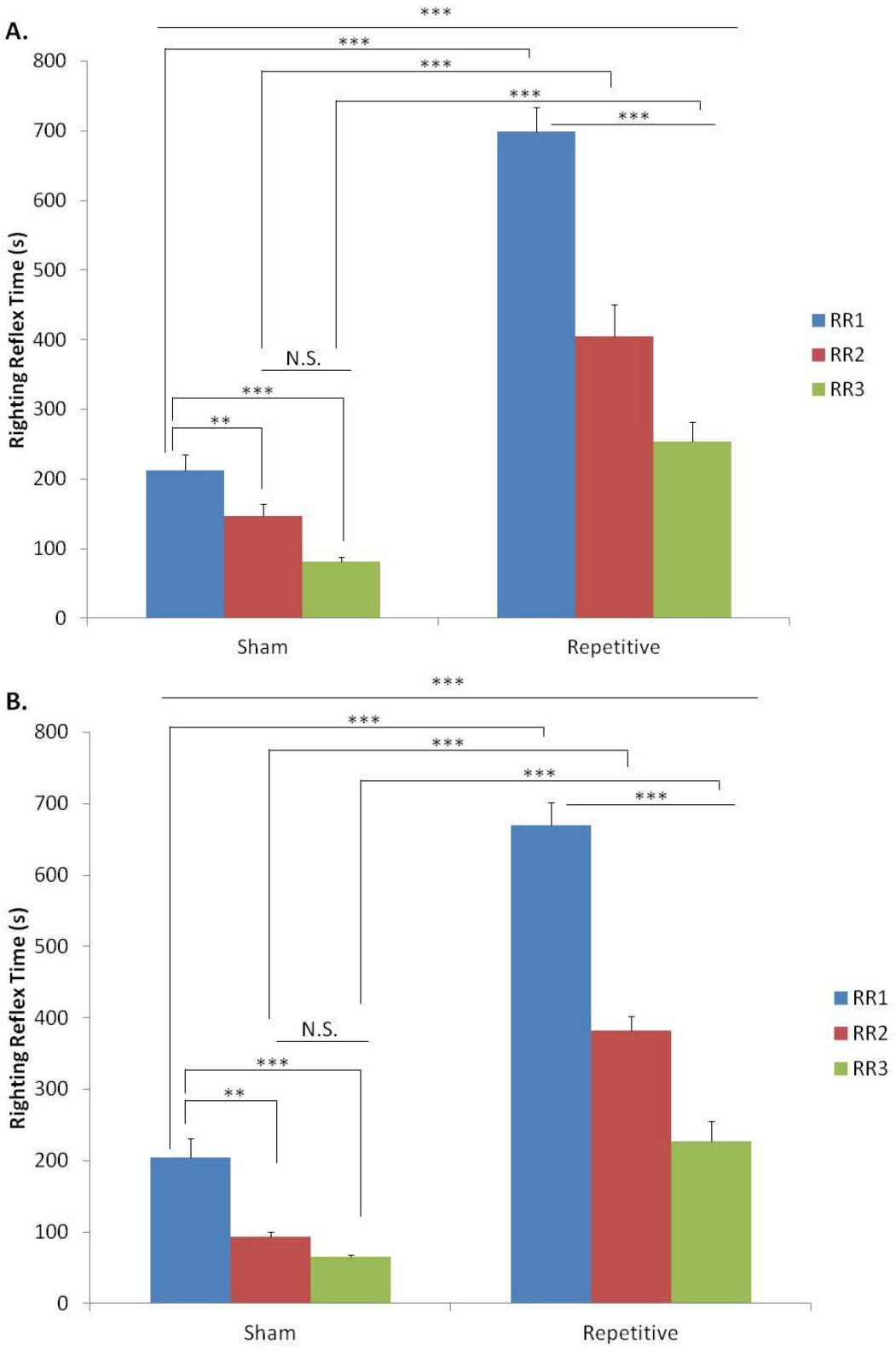

A.
Righting Reflex Time (s)
800
700
600
500
400
300
200
100
0
***
***
***
***
***
N.S.
***
**
Sham
Repetitive
RR1
RR2
RR3
B.
Righting Reflex Time (s)
800
700
600
500
400
300
200
100
0
***
***
***
***
***
N.S.
***
**
Sham
Repetitive
RR1
RR2
RR3

Figure 3.3

Figure 3.4: Depressive behavior decreases in repetitively brain-injured mice. Depressive-like behavior was evaluated using the forced swim test in male (A) and female (B) sham injured and brain-injured mice. In male mice, repeated measures ANOVA indicated a decrease in depressive-like behavior between sham injured and brain-injured male mice with no effect of time ($p < 0.01$). Additionally, while there was a significant effect of injury, there was no effect of exercise. In female mice, repeated measures ANOVA also indicated a decrease in depressive-like behavior between sham injured and brain-injured female mice ($p < 0.05$). Again, while there was a significant effect of injury, there was no effect of exercise. Figures are presented as group mean ± Standard Error of the Mean (SEM); *$p < 0.05$, **$p < 0.01$

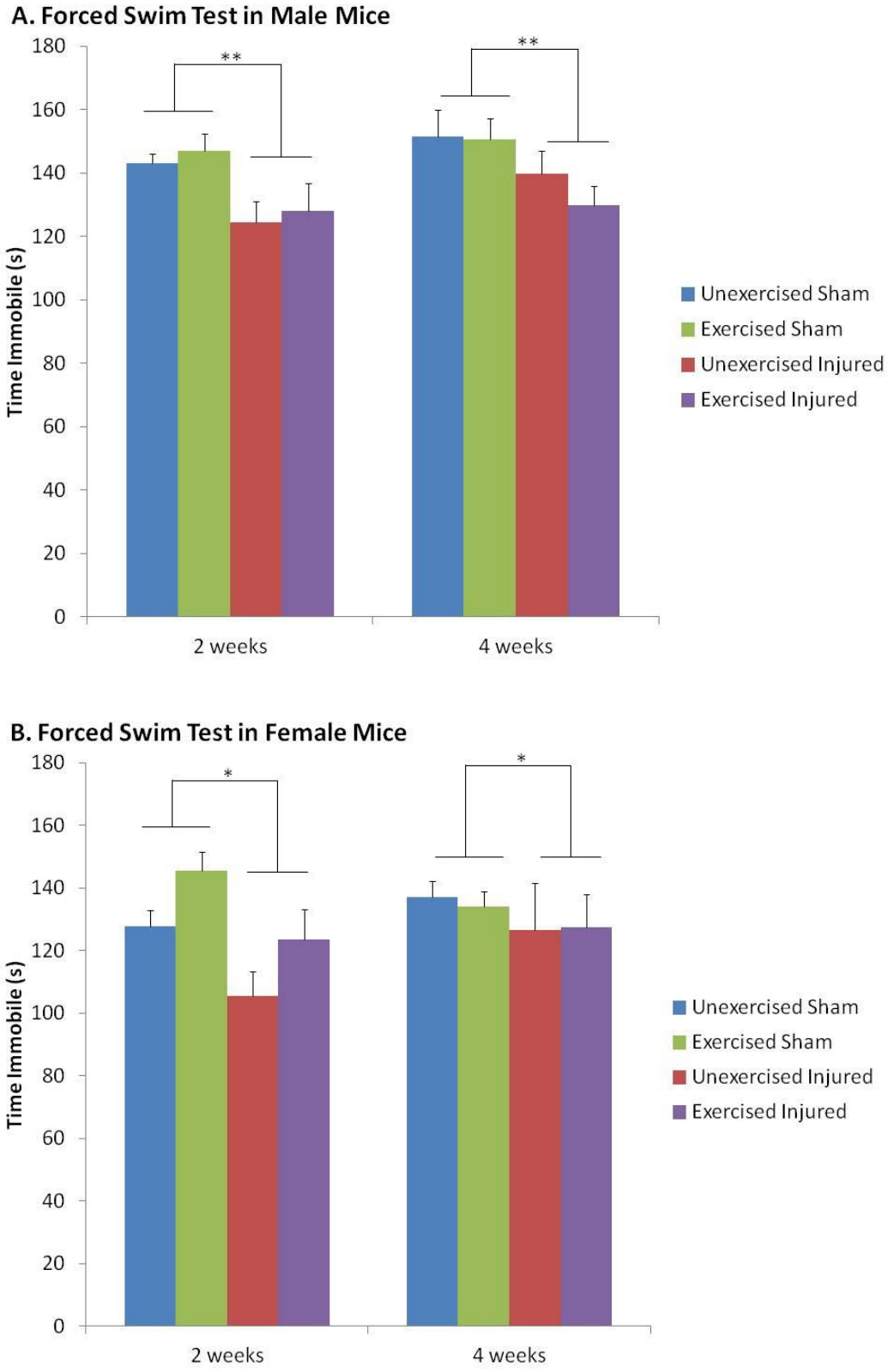

A. Forced Swim Test in Male Mice
Time Immobile (s)
180
160
140
120
100
80
60
40
20
0
**
**
2 weeks
4 weeks
Unexercised Sham
Exercised Sham
Unexercised Injured
Exercised Injured
B. Forced Swim Test in Female Mice
Time Immobile (s)
180
160
140
120
100
80
60
40
20
0
*
*
2 weeks
4 weeks
Unexercised Sham
Exercised Sham
Unexercised Injured
Exercised Injured

Figure 3.4

Figure 3.5: Periorbital mechanical sensitivity decreases over time in brain-injured male mice. At 2 and 4 weeks post-injury or post-surgery, mice were evaluated for changes in periorbital mechanical sensitivity. Repeated measures ANOVA indicated an overall increase in periorbital threshold indicative of decreased sensitivity at 4 weeks compared to 2 weeks ($p < 0.05$) as well as an increase in threshold of brain-injured male mice between 2 weeks and 4 weeks ($p = 0.004$) that is not observed in sham injured male mice (A). No effect of exercise was observed. Percent of male mice displaying allodynic thresholds is presented in (B). A higher percentage of brain-injured male mice display allodynia at 2 weeks compared to sham injured mice for both unexercised ($p = 0.049$) and exercised ($p = 0.033$) mice, and percentage decreases in all mice at 4 weeks compared to 2 weeks but is not significantly different at this time. Figures are presented as group mean ± Standard Error of the Mean (SEM); **$p < 0.01$

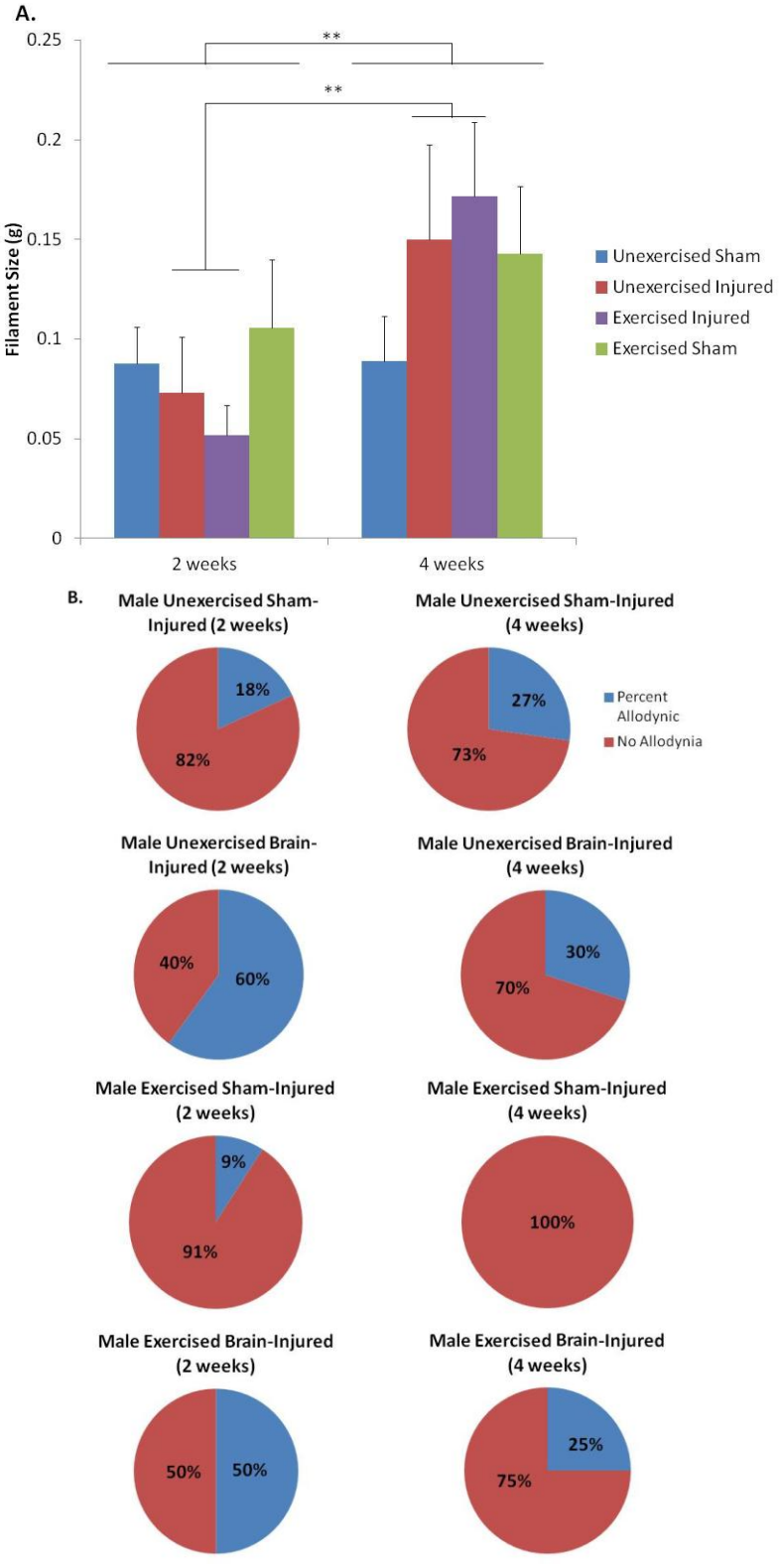
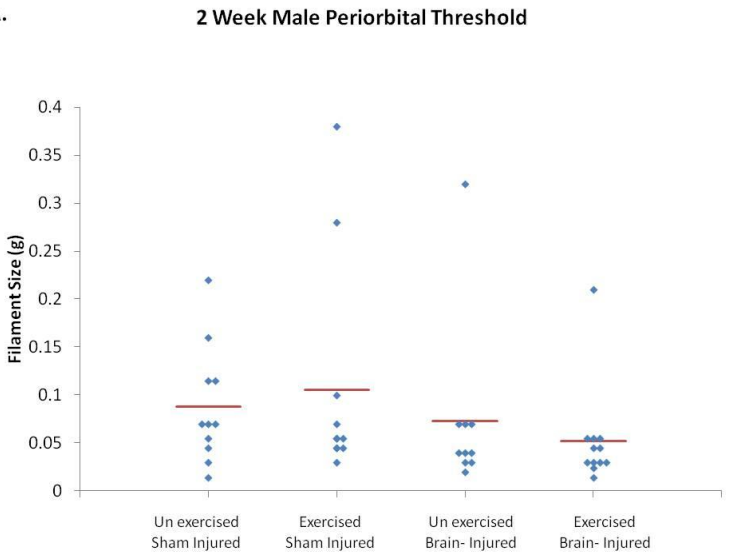

A.
0.25
0.2
0.15
0.1
0.05
0
Filament Size (g)
**
**
2 weeks
4 weeks
Unexercised Sham
Unexercised Injured
Exercised Injured
Exercised Sham
B.
Male Unexercised Sham-Injured (2 weeks)
18%
82%
Male Unexercised Sham-Injured (4 weeks)
27%
73%
Percent Allodynic
No Allodynia
Male Unexercised Brain-Injured (2 weeks)
40%
60%
Male Unexercised Brain-Injured (4 weeks)
30%
70%
Male Exercised Sham-Injured (2 weeks)
9%
91%
Male Exercised Sham-Injured (4 weeks)
100%
Male Exercised Brain-Injured (2 weeks)
50%
50%
Male Exercised Brain-Injured (4 weeks)
25%
75%

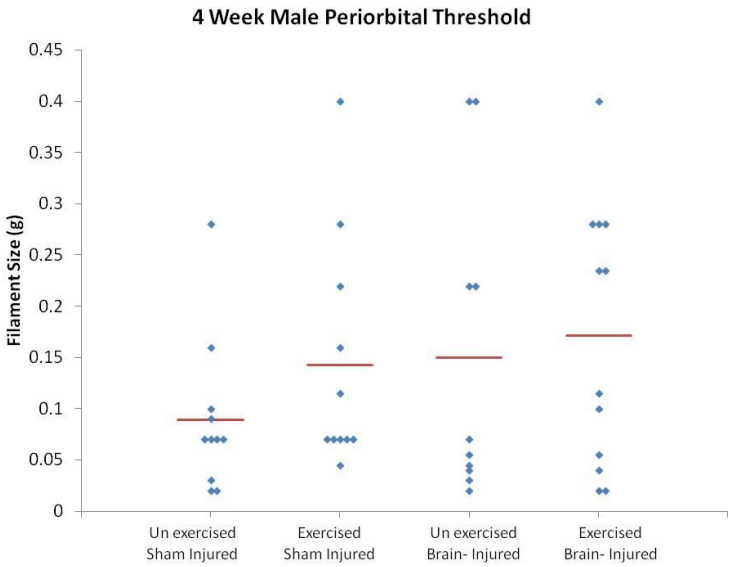
Figure 3.5

Figure 3.6: Distribution of periorbital threshold in male mice. Periorbital threshold for male exercised and unexercised sham injured and brain-injured mice is given for 2 weeks (A) and 4 weeks (B). Means are displayed as a horizontal line for each group. A Kruskal-Wallis analysis indicated a significant difference between exercised sham injured and brain-injured male mice ($p = 0.036$). Values from Mann-Whitney U analyses are presented in (C). NS = not significant

A.



B.



C. Male Mice	2 weeks		4 weeks	
	U value	p value	*U* value	p value
Unexercised sham injured versus brain-injured	37.5	NS	50.5	NS
Exercised sham injured versus brain-injured	32.5	**0.038**	61.5	NS
Sham injured exercised versus unexercised	54.0	NS	43.0	NS
Brain-injured exercised versus unexercised	48.0	NS	51.0	NS

Figure 3.6

Figure 3.7: Periorbital mechanical sensitivity increases over time in unexercised female mice. At 2 and 4 weeks post-injury or post-surgery, mice were evaluated for changes in periorbital mechanical stimulation. Repeated measures ANOVA indicated a decrease in periorbital threshold ($p = 0.02$) indicative of increased sensitivity in threshold of unexercised female mice between 2 weeks and 4 weeks that is not observed in exercised female mice (A). Percent of female mice displaying allodynic thresholds is presented in (B). A higher percentage of brain-injured female mice display allodynia at 2 weeks compared to sham injured mice regardless of exercise status, and a higher percentage of exercised mice develop allodynia compared to unexercised mice. Percentage increases in unexercised mice and exercised brain-injured mice at 4 weeks compared to 2 weeks and decreases in exercised sham injured female mice. However, no changes in percentages of groups displaying allodynic thresholds were significant ($p > 0.05$). Figures are presented as group mean ± Standard Error of the Mean (SEM); $*p < 0.05$

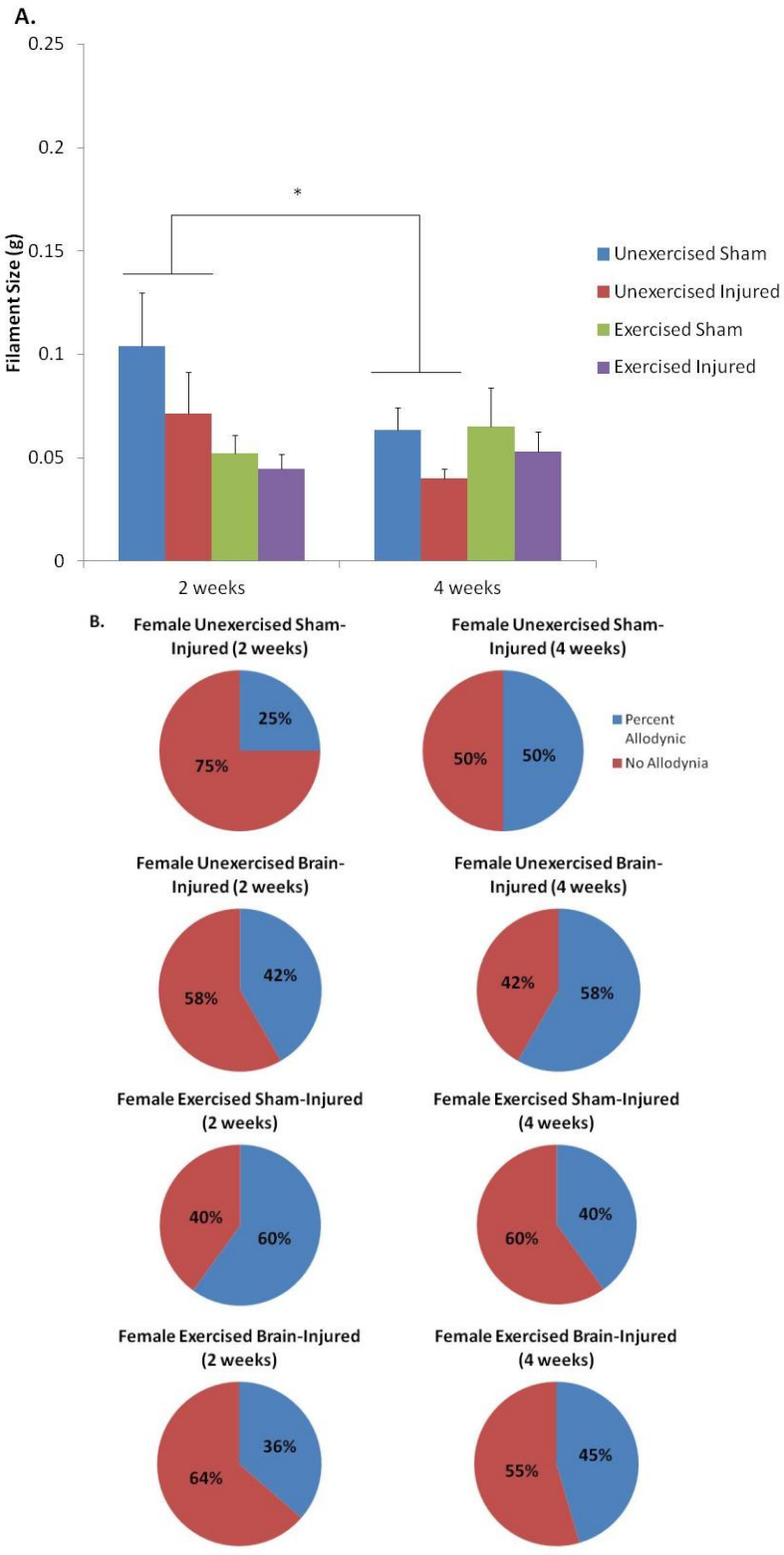
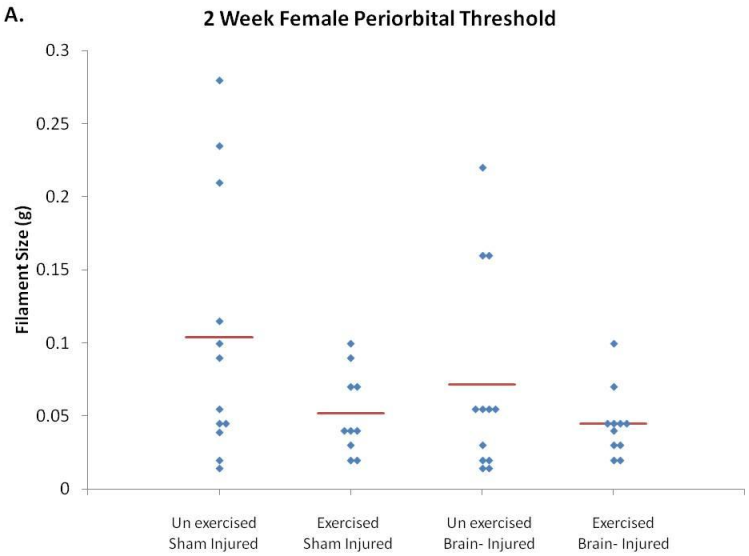
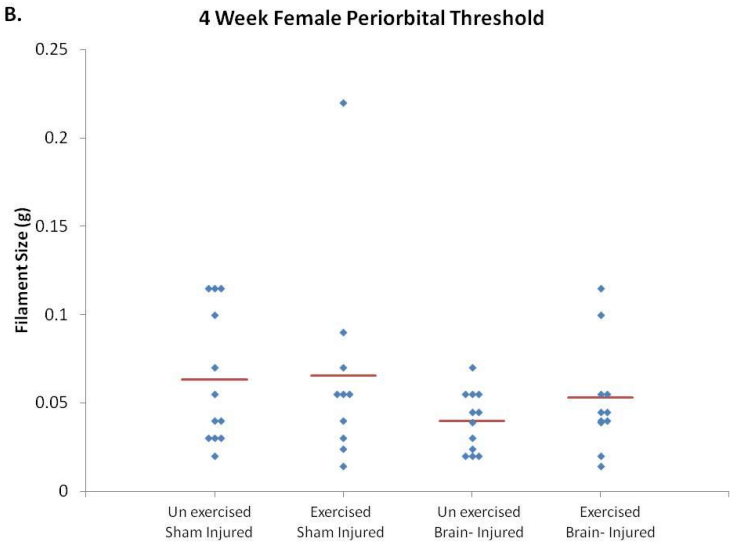

A.
0.25
0.2
0.15
0.1
0.05
0
Filament Size (g)
*
Unexercised Sham
Unexercised Injured
Exercised Sham
Exercised Injured
2 weeks
4 weeks
B.
Female Unexercised Sham-Injured (2 weeks)
25%
75%
Female Unexercised Sham-Injured (4 weeks)
50%
50%
Percent Allodynic
No Allodynia
Female Unexercised Brain-Injured (2 weeks)
42%
58%
Female Unexercised Brain-Injured (4 weeks)
42%
58%
Female Exercised Sham-Injured (2 weeks)
40%
60%
Female Exercised Sham-Injured (4 weeks)
60%
40%
Female Exercised Brain-Injured (2 weeks)
36%
64%
Female Exercised Brain-Injured (4 weeks)
55%
45%

Figure 3.7

Figure 3.8: Distribution of periorbital threshold in female mice. Periorbital threshold for female exercised and unexercised sham injured and brain-injured mice is given for 2 weeks (A) and 4 weeks (B). Means are displayed as a horizontal line for each group. A Kruskal-Wallis analysis indicated no significant differences between groups ($p > 0.05$). Values from Mann-Whitney U analyses are presented in (C). NS = not significant





C. Female Mice	2 weeks		4 weeks	
	U value	p value	*U* value	p value
Unexercised sham injured versus brain-injured	55.5	NS	48.0	NS
Exercised sham injured versus brain-injured	52.0	NS	48.5	NS
Sham injured exercised versus unexercised	40.0	NS	55.5	NS
Brain-injured exercised versus unexercised	58.0	NS	56.0	NS

Figure 3.8

CHAPTER 4

SUMMARY OF FINDINGS, CONCLUSIONS, AND FUTURE DIRECTIONS

4.1 SUMMARY OF FINDINGS

These studies have begun to illuminate common understudied symptoms of mTBI including emotional symptoms of depression and physical symptoms of headache. I examined these symptoms following a single mTBI or rmTBI in both male and female mice as well as including exercised or unexercised mice for rmTBI. For a single injury, I followed a previously published model of TBI that has been shown to produce transient working memory deficits and cellular pathology consistent with mTBI.

First, I injured male and female mice with a single mTBI and evaluated them for development of depressive-like behavior and periorbital allodynia. At the conclusion of behavioral testing, a subset of mice underwent voltammetry experiments to evaluate concentrations of extracellular DA and *tau* at baseline and following DAT inhibition. I found that male brain-injured mice exhibited an increase in depressive-like behavior in the FST but not the TST, and in addition, these mice showed a significantly higher amount of extracellular DA following DAT inhibition than sham-injured male mice and brain-injured female mice. Taken together, this suggests that altered DA neurotransmission in the mesocorticolimbic pathway of male brain-injured mice that may be related to emotional symptoms of mTBI. This is consistent with a previously proposed mechanism of the involvement of DA dysfunction in depression in which decreased available DA leads to compensatory mechanisms such as increased postsynaptic DA receptor density and decreased DAT number or function, ultimately leading to an increase in DA signal transduction.

I also observed an increase in periorbital sensitivity in female brain-injured mice following a single mTBI. This is indicative of the development of allodynia as a model of

PTH, something that has been reported both clinically and preclinically. However, while I observed this in female brain-injured mice, no change in periorbital sensitivity was observed in male mice, contrary to previous reports. This may be due to injury severity, as previous preclinical publications in mice have only used moderate to severe injury and have only examined male animals. My results demonstrate the importance of both injury severity and sex in development of post-traumatic pain.

I also evaluated these symptoms following rmTBI in exercised and unexercised mice of both sexes. Contrary to my findings in single mTBI, I observed a significant decrease in depressive-like behavior in all brain-injured mice regardless of sex or exercise status. It is possible that these contrary findings are methodological, resulting from repeated exposure to anesthesia that may have provided a protective effect against injury or altered behavior through isoflurane-induced changes to neurochemistry. Alternatively, it is possible that the decrease in depressive-like behavior may be an injury effect indicative of locomotor hyperactivity. Additional behavioral tests are necessary to differentiate between these options or introduce supplemental explanations.

Finally, I observed a decrease in periorbital sensitivity in male brain-injured mice that may suggest hyposensitivity in these mice. Additionally, I observed an increase in periorbital sensitivity in unexercised female mice at 4 weeks compared to 2 weeks but no significant differences between exercised and unexercised mice or between sham injured and brain-injured female mice. This is likely due to limitations in the sensitivity of the test, and additional tests for periorbital sensitivity with greater sensitivity may reveal differences that I could not observe here. However, these results support the sex-

dependent effects of rmTBI on development of allodynia as well as the influence of exercise on post-traumatic symptoms.

Taken together, these data indicate a sex-specific response to emotional and physical symptoms of depression and PTH as well as a sex-specific response of the exercised brain to rmTBI. While additional tests are necessary to elucidate the effects observed here, this provides the basis for continued exploration of the effects of exercise on mTBI after single or repeated injury in both male and female mice, contributing to the development of more relevant models for athlete TBI.

4.2 CONCLUSIONS

The effects of mTBI and rmTBI on development of mood and pain disorders have been well documented, but the effect of exercise on response to TBI is understudied and its importance at various times pre-, post-, or during injury are unclear. Additionally, the effect of sex or gender on these symptoms is still poorly understood despite clinical evidence of gender-specific risks of PCS and incidence of specific post-traumatic symptoms. Emotional symptoms, especially depression, are gaining attention in the media and scientific community due to their increased prevalence in athlete populations, and a high amount of debate exists as to the timing of exercise post-injury. However, the preclinical literature regarding these questions is incomplete, particularly in terms of exercise prior to injury, reducing the relevance of these studies to the clinical population. The studies performed here address not only the question of sex-specific development of depression and PTH and an investigation into potential mechanisms but also begin to elucidate exercise as an important factor in the response of the brain to rmTBI.

While this research provides only the beginning of an understanding of these effects, it significantly contributes to the field of athlete TBI. I evaluated understudied symptoms of depression and PTH in an animal model of mTBI. I also developed a model of athlete TBI that mimics the exercised condition of the athlete in mice, a model that is lacking in current preclinical research. These studies will provide the basis for continuing studies in a more relevant model of athlete rmTBI, ultimately leading to improved guidelines for post-injury treatment of athletes.

4.3 FUTURE DIRECTIONS

While these studies demonstrate sex-specific responses to both a single mTBI and rmTBI as well as a sex-specific effect of exercise on symptoms following injury, they also raise a number of questions and open new avenues for exploration in future studies. One of these questions is to continue to understand the role of altered DA neurotransmission following not only single mTBI but also rmTBI, particularly as it may relate to onset and persistence of depressive symptoms, using both in vivo methods and immunohistochemistry. Another question is to continue to investigate the development of allodynia in female mice that is not observed in male mice.

Beyond the questions of the cellular and functional changes that may lead to debilitating emotional and physical symptoms in the clinical population, there are also a number of questions related to the influence of exercise on outcome following mTBI and rmTBI. While exercise prior to injury increases the ability of this athlete model to translate from mouse to human studies, it lacks realistic post-injury exercise. One of the biggest questions remaining among clinicians in treating athletes following mTBI is how long of a rest period is required for the most recovery before returning to play. While a 2 week period of both mental and physical rest is typically recommended, this remains a controversial topic. Clinicians recommend varying levels of activity and length of rest period, such as a gradual return to pre-injury activity versus strict rest during the rest period, and some researchers have suggested that strict rest following TBI in athletes may be detrimental due to a withdrawal of trophic factors from the site of injury when activity ceases (Semple et al., 2015, Thomas et al., 2015). To evaluate these questions, the pre-injury exercise used in the current study may be combined with varying levels of post-

injury activity including but not limited to a 2 week period of strict rest or a 2 week period of rest including a gradual return to activity. Additionally, in the context of rmTBI, an additional question addresses activity between injuries. While it is recommended that athletes who sustain a head injury do not return to play on the same day as the injury and some sports leagues require clearance by a team doctor or neurological consultant before returning to play, symptoms of mTBI are not always reported by athletes; as a result, athletes with mTBI may return to play before resolution of symptoms or during vulnerable periods in which a subsequent injury may exacerbate initial symptoms (Didehbani et al., 2013). To address this possibility, exercise may be continued at either pre-injury intensities or at a milder intensity between injuries as well as following the final injury or after the 2 week rest period. Addressing these questions may lead to a greater understanding of how exercise immediately following injury coupled with an exercised condition may influence symptom outcome, providing a basis for modifying treatment guidelines among athletes.

In conclusion, while this study begins to illuminate differences in symptoms following mTBI or rmTBI and responses of the exercised brain versus the unexercised brain to rmTBI in males and females, these studies only begin to examine these effects. Future studies will continue to provide valuable information regarding depression and PTH after these types of mild injury and may ultimately lead to improved gender-specific clinical guidelines and treatments for athletes.

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